Studies on the Control of the Citric Acid Cycle in Heart

by

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May, 1977

This thesis was written by me and the work described therein was carried out entirely by me.

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Abstract

Since the acceptance of the Citric Acid Cycle as the major route of oxidation in almost all tissues, considerable interest has been focussed on the control of this most fundamental metabolic pathway. This thesis describes studies carried out to investigate the control of the Citric Acid Cycle in heart muscle, using a computer simulation model of the pathway. The simulation technique used was that known as CHEK. This is a program designed for digital computer simulation which is not only rapid and easy to handle, but also allows the simulation of a large number of chemical reactions such as those describing a complex metabolic pathway.

The simulation model of the Citric Acid Cycle was constructed by formulating models of each of the component enzymes of the Cycle from information available in the literature and joining these together. Published values for the concentrations of enzymes, coenzymes and intermediates, and ratios of the various forms of the coenzymes were also used.

To assess the control features of the Cyclic enzymes in the simulation studies, the concepts of Sensitivity (control strength) and Elasticity (effector strength) were employed.

It was found that, of the Cycle enzymes, citrate synthase (EC: 4.1.3.7) was the only enzyme whose activity exhibited significant control over Cycle flux under the conditions used for the simulations. This was due to the fact that this enzyme was almost saturated with acetylCoA under these conditions. When a 'dummy' reaction representing the input of acetylCoA was included in the simulation model, it was noted that Citric Acid Cycle flux was dependent to a greater or lesser extent on the rate of this reaction (i.e. on the steady state level of acetylCoA).
As the rate was decreased this 'enzyme' became increasingly 'Sensitive' (i.e. a more important site of regulation) while the control properties

of citrate synthase diminished greatly. This situation was reversed as the rate of acetylCoA production was increased. These phenomena can be explained in terms of the saturation of citrate synthase by acetylCoA.

Variations in NADH concentration were found to exhibit marked effects on Citric Acid Cycle flux. Further investigation showed that this was a result of altering the equilibrium distribution of the substrates and products of malate dehydrogenase (EC.1.1.1.3), thus causing an alteration in the level of oxalacetate, which in turn affected the flux through citrate synthase and hence Cycle flux. A 'dummy' reaction representing the oxidation of NADH by the electron transport system was included in the simulation model. As expected from the previous studies, this exhibited very sensitive control of Cycle flux under almost every set of conditions used for the simulation studies, and appeared to be the major site of Cycle regulation.

The suggestion that sequestration of oxalacetate might have a 'controlling' effect in the Citric Acid Cycle was not supported by the simulation studies. Due to the very high equilibrium constant of the malate dehydrogenase reaction and the fact that the enzyme was close to equilibrium during Cycle operation, any oxalacetate removed from the system was almost immediately replenished from the large pool of malate, causing very little change in the steady state Cycle flux.

The findings described in the previous paragraphs lead to the conclusion that control of the Citric Acid Cycle depends on a number of factors: the activity of citrate synthase, the equilibrium nature of the malate dehydrogenase reaction and the high value of K_{eq} for this reaction, the rate of production (and hence the steady state concentration) of acetylCoA and the rate of re-oxidation of NADH. Although each of these factors alone would be important to the control of the Cycle, together they form a much more efficient and extremely sensitive mechanism of Cycle

regulation. This regulation not only affords a means whereby supply and demand are highly integrated, but also provides a flexible control which will operate under a very wide variety of conditions.

The utilization of acetoacetate by the Citric Acid Cycle in heart muscle was also investigated. It was found that unless the reaction of succinyl thiokinase (EC.6.2.1.4) was retarded in some way (and hence the steady state concentration of succinylCoA raised), the acetoacetate 'activation' pathway could not operate. The system was made to function by employing a much higher GTP:GDP ratio than had been previously used. This raised the problem of the relationship between the phosphorylation states of the adenine and guanine nucleotides and the functioning of the enzyme nucleoside diphosphate kinase (EC.2.7.4.6). The control parameters of the Cycle enzymes were calculated where there were high ratios first of GTP:GDP, and then of GTP:GDP and ATP:ADP; in each case the effects of acetylCoA or acetoacetate as the carbon source for the Cycle were examined. A simulation of nucleoside diphosphate kinase was also included in the Citric Acid Cycle model. These investigations led to no definite conclusions about the situation existing when acetoacetate is the major source of acetylCoA for the Cycle. Much more experimental investigation, particularly with regard to the ratios of ATP:ADP and GTP:GDP within the heart mitochondria, the relationship between these and the operation of nucleoside diphosphate kinase, will be required before any further comprehension of the utilization of ketone bodies by the heart can be made. It was found that a high ratio of GTP:GDP with or without an accompanying high ratio of ATP:ADP made no alteration to the conclusions previous reached concerning the control of the Citric Acid Cycle.

The thesis also contains a report of an experimental investigation of the kinetic properties of 2-oxoglutarate dehydrogenase system. Initial velocity studies were carried out, the results of which did not agree with the Hexi Uni Ping Pong mechanism previously proposed for this enzyme complex. The results were analysed by fitting the initial velocities

to rate equations describing five different reaction mechanisms by means of an optimization procedure. Quantitative and qualitative evaluation of the optimization analysis indicated that one mechanism, in which there is a random binding of NAD^+ and CoA and release of succinylCoA, gave a significantly better fit to the data than others. Rate constants for the partial reactions of this mechanism were calculated from the data gained from the optimization study. These were used, with the mechanism suggested by the experimental results, in the simulation model of the Citric Acid Cycle in place of the previously used Hexa Uni Ping Pong mechanism. Simulation studies using the 'new' model for 2-oxoglutarate dehydrogenase indicated that there were no significant alterations of the conclusions drawn from previous simulations containing the 'old' model for 2-oxoglutarate dehydrogenase.

Abbreviations Used

AcetoacetylCoA	:	acetoacetyl coenzyme A
AcetylCoA	:	acetyl coenzyme A
ADP	:	adenosine diphosphate
AMP	:	adenosine 5' monophosphate
ATP	:	adenosine triphosphate
CoA	:	coenzyme A
Cyclic AMP	:	3'5' cyclic adenosine monophosphate
FAD	:	flavin adenine dinucleotide
GDP	:	guanosine diphosphate
GTP	:	guanosine triphosphate
NAD^+	:	nicotinamide adenine dinucleotide
NADH	:	reduced NAD^+
$NADP^+$	:	nicotinamide adenine dinucleotide phosphate
NADPH	:	reduced $NADP^+$
P_i	:	phosphate
SuccinylCoA	:	succinyl coenzyme A
TPP	:	thiamine pyrophosphate.

CHAPTER I

INTRODUCTION

i) History of the Citric Acid Cycle

The Citric Acid Cycle is the pathway occurring in all aerobically respiring cells (Krebs & Lowenstein, 1960) which acts as the mechanism for extracting the energy from chemical intermediates after preliminary degradations are complete and passes it on to the ATP - producing respiratory chain as reducing equivalents which are eventually oxidised by a terminal electron acceptor. The entire process of oxidation puzzled the biological world for a very long time, though in retrospect it is obvious that the elucidation of such a complex series of events was not a problem to be easily solved, and many of the finer details of mechanism and control are still not fully understood.

Although the history of the discovery of the Citric Acid Cycle spans a period of about three centuries, real progress dates from the beginning of this century. The fact that respiration occurs in the tissues had been accepted since the mid-nineteenth century (e.g. von Liebig, 1850; Bernard, 1876), but further useful research only came after the downfall of the 'Protoplasmic' theory of cellular metabolism. Protoplasm was for long considered to be one entity, comprising the entire intracellular space, which 'biologically' carried out all the functions of the cell. With Buchner's (1897) demonstration of cell-free fermentation, it was realised that metabolism is a chemical process catalysed by enzymes which could be chemically distinguished by chemical means (Buchner & Rapp, 1897, 1899). Research could then advance on a sound scientific basis. The work of Thunberg (1909, 1910) and Batelli & Stern (1911) who demonstrated, by chemical means the oxidation of succinate in tissue extracts, finally discredited the Protoplasmic theory and the 'Vitalist' school fell

into decline. This work in fact was the first part of the Citric Acid Cycle to be discovered. Because of the tight coupling of the oxidation of succinate to the electron transport chain, the discovery was important to the later elucidation of both the Cycle and the respiratory chain.

Much research had been devoted to the mechanism of respiration at the molecular level. There were two conflicting theories. Warburg, on the basis of his experiments on the iron-containing 'Atmungsferment' (now known as cytochrome oxidase) which has oxygen transporting properties, proposed that cellular respiration was accomplished by oxygen (Warburg, 1925). Meanwhile, Wieland developed an apparently opposing theory. He believed that biological oxidations were catalysed by hydratase (dehydrogenase) enzymes, the activated hydrogens being passed on to a suitable acceptor such as quinone, methylene blue or oxygen (Wieland, 1922). There was much heated controversy over these two views but as Hopkins (1926) pointed out they were mutually exclusive only if a very narrow view of either was taken. The situation was finally resolved by Keilin (1928) in his classic work on the role of the cytochromes in respiration. Keilin proposed that oxidation took place in two stages, the reduction of cytochrome by the dehydrogenase systems and the oxidation of reduced cytochrome by molecular oxygen.

Research on the stages of respiration prior to the cytochrome chain was also progressing. Einbeck (1919) demonstrated the activity of the enzyme fumarase (the name 'fumarase' was coined by Batelli & Stern (1921)), as distinct from succinic dehydrogenase, although both of these activities had been indicated by the earlier work of Thunberg (1910) and Batelli & Stern (1911).

Malate dehydrogenase activity was later characterised by Green and his colleagues (see Green, 1936). This was an important advance in the work on the mechanisms of oxidation since it demonstrated the link between the substrate dehydrogenations and the respiratory chain, thus supporting the theory first proposed by Keilin (1928). Malate dehydrogenase was later to become an important part of Krebs' proposal of a cyclic oxidation of citric acid.

Needham (1927) demonstrated that muscle tissue oxidises succinate to fumarate to malate and concluded from her experiments that in muscle there was continuous formation and removal of succinate going on in the tissue. This perhaps was the first insight into the catalytic function of these carboxylic acids in oxidation, though it took a number of years for this concept to be fully appreciated.

Szent-Györgyi had become interested in the controversy of Wieland and Warburg, and using succinate he also demonstrated that oxidation involved both 'oxidation' and dehydrogenation. His later work on the importance of '4-carbon metabolites' stemmed from these studies as he believed that such intermediates played an important role in biological oxidation. The culmination of these studies was the publication in 1935 of his theory of fumarate catalysed respiration. This theory was subsequently adopted to fit in with what was known of the glycolytic pathway (Szent-Györgyi, 1937), so that his scheme of respiration could be considered as an oxidation of trioses by oxalacetate. The importance of this theory was the consideration of the 4-carbon carboxylic acids as part of the oxidation system rather than as muscle fuels. Also by taking triose as the starting material for respiration, glycolysis and respiration were linked together as two parts of one system, a fundamental idea to the understanding of the integration of cellular metabolism.

A similar observation about the role of the 4-carbon metabolites was made by Stare & Baumann (1936) who noted the catalytic effect of fumarate on respiration. On adding fumarate to minced tissue they found six to ten times as much oxygen was taken up than fumarate alone would have needed for its complete oxidation. They concluded that fumarate acts primarily as a catalyst for respiration, and at high concentrations it also acts as a substrate.

The next part of the Cycle came to light as a result of the demonstration by Martius (Martius & Knoop, 1937; Martius, 1937) that succinate could be formed from citrate. Although this work was originally intended to show that citrate was a source of pyruvate and hence a fuel for oxidation, it played an important part in the later formulation of Krebs' theory of the Citric Acid Cycle (Krebs & Johnson, 1937b). Martius was the first to demonstrate the separate activities of the enzymes aconitase and isocitrate dehydrogenase. Previously these had been designated as one system called 'Citric dehydrogenase' (Bernheim, 1928). It was Breusch (1937) who coined the name aconitase for the system which he showed could interconvert the acids citrate, cis-aconitate and isocitrate.

The oxidation of 2-oxoglutarate to succinate and CO_2 was first noted by Weil-Malherbe (1937) who used malonate to prevent any further oxidation of the succinate. Krebs & Johnson (1937a) also reported this activity in kidney and liver tissue. Later Ochoa (1944) investigated the activity of 2-oxoglutarate dehydrogenase in cat heart tissue and showed the reaction to be:

$$\text{2-oxoglutarate} + \tfrac{1}{2}O_2 \rightleftharpoons \text{succinate} + CO_2$$

He suggested that the reaction was coupled to oxidative phosphorylation via a flavin nucleotide and demonstrated that three molecules of ATP were produced in the process. It was not until much later that it

was realised that this conversion was brought about by two enzymes viz: 2-oxoglutarate dehydrogenase and succinyl thiokinase. The 2-oxoglutarate dehydrogenase was purified by Sanadi and co-workers (1951; 1952) who identified the products of the enzymic reaction as NADH, succinylCoA and CO_2. At about the same time Kaufman discovered the activity of 'Phosphorylating' enzyme (now known as succinyl thiokinase) (Kaufman, 1951) and went on to characterise the enzymic reaction (Kaufman et al., 1953).

The last link in the chain was supplied by Krebs & Johnson (1937b) who demonstrated that citrate could be formed from oxalacetate plus a 'triose', in minced pigeon breast muscle. Taking this together with the work of Szent-Györgyi, Stare & Baumann and Martius & Knoop, they proposed a cycle in which the starting material, oxalacetate, is regenerated after the degradations of the Cycle have taken place. The idea of a cyclic sequence of events was perhaps the most elegant feature of Krebs' theory though it was not entirely a new concept since he had proposed the Urea Cycle five years previously (Krebs & Henseleit, 1932).

In his original theory Krebs could not show which metabolite supplied the 2-carbon fragment which reacted with oxalacetate to form citrate, although he suggested that pyruvate, acetate or some phosphate ester (from glycolysis) were possibilities. In a subsequent publication (Krebs, 1937) he tended to emphasize pyruvate as the precursor. He also pointed out that pyruvate gives rise to ketone bodies, and suggested that pyruvate had an important role at the branch point of these pathways.

Isolation and characterisation of the enzyme catalysing the initiating condensation reaction of the Cycle came much later than the demonstration of its existence by Krebs & Johnson. It was not until 1950 that the enzyme was purified enough for its substrates and products to be identified (Novelli & Lipmann, 1950; also Ochoa et al., 1951; Stern et al., 1952).

Perhaps the very simplicity of the Citric Acid Cycle aroused suspicions about its role as the major route of biological oxidation. In the years following 1937 there were many publications supporting the validity of the Cycle (e.g. Krebs et al., 1938; Banga et al., 1939; Hallman & Simola, 1939; Smyth, 1940; Evans, 1941), but there was also fairly strong opposition to the proposal that it was the preferential pathway of carbohydrate oxidation in the tissues, from various workers among them Baumann & Stare (1940) upon whose earlier work Krebs' proposal of the Citric Acid Cycle was partly based (Baumann & Stare, 1940; Stare et al., 1941). There was also a school of thought which continued to support Szent-Györgyi's pathway (Breusch, 1937; 1939; Thomas, 1937). Krebs did not take these criticisms very seriously and countered them with his own criticisms of the Szent-Györgyi theory (Krebs, 1943).

Radioactive tracer experiments carried out by Wood et al. (1941) and Evans & Slotin (1941) suggested that Krebs' original sequence of production of the tricarboxylic acids was erroneous; the labelling patterns suggested that cis-aconitate was the first product of the condensation of oxalacetate with the triose precursor. Krebs himself agreed with these findings (Krebs, 1943) and amended the Cycle accordingly. This new sequence of reactions was accepted until 1948 when Ogston (1948) pointed out that a symmetrical molecule can undergo a non-symmetrical reaction when the catalyst is an enzyme.

Proof of this for the condensation reaction soon followed (Potter & Heidelberger, 1949; Wilcox et al., 1950; Lorber et al., 1950; Martius & Schorre, 1950). Krebs reviewed the findings in detail some time after the controversy had been settled (see Krebs & Lowenstein, 1960).

While the arguments as to the existence and importance of the Cycle continued, work was progressing in two other areas of metabolism which were to have great importance in relation to the Cycle, the oxidation of pyruvate and the oxidation of fatty acids. The elucidation of the former dates from the observation of Kinnersly and Peters (1929) that a thiamine deficiency brings about an accumulation of lactate. It was shown that this was not due to any alteration in the rate of glycolysis (Fisher, 1931) and that under deficiency conditions there is a reduction of O_2 uptake (Gavrilescu et al., 1932). Banga,Ochoa & Peters (1939) later demonstrated that the active form of the vitamin was the pyrophosphate ester and that brain tissue from a deficient animal would only oxidise pyruvate if thiamine pyrophosphate was added.

At about this time, Lipmann was working on acetylation reactions. Having demonstrated in certain micro-organisms that 'active acetate' was in fact acetyl phosphate (Lipmann & Tuttle, 1945), he turned his attention to animal systems where it was already known (Klein & Harris, 1938) that acetylation is linked to respiration. He could not confirm that acetyl phosphate was the 'active acetate' in animal systems but he did partially purify a coenzyme of acetylation from pigeon liver extract. This was shown to contain pantothenic acid which is linked through a thioester bond to the acetyl residue (Lynen & Reichert, 1951). This was given the name of coenzyme A (coenzyme for acetylation) by Lipmann et al. (1947). The demonstration that acetyl CoA was the elusive 2-carbon fragment which reacted with oxalacetate in the

initiating reaction of the Cycle soon followed (Novelli & Lipmann, 1950; Stern et al., 1952).

The last remaining link, was the demonstration that acetylCoA was formed from pyruvate. Working with E.coli, Korkes et al. (1951) partially purified a system which catalysed the reaction:

$$\text{pyruvate} + NAD^+ + CoA \rightleftharpoons \text{acetylCoA} + NADH + H^+ + CO_2$$

The elucidation of the pathway of catabolism of fats and particularly of the fatty acids was as controversial and complex as the Citric Acid Cycle itself. The connection between these two pathways became apparent as the elucidation of both mechanisms progressed.
In 1939, Leloir & Munoz demonstrated that fumarate accelerated fatty acid oxidation and diminished the production of ketone bodies in a cell free system. At this time there was great controversy as to whether the product of fatty acid oxidation was acetoacetate or acetate, however this was settled by the work of Weinhouse, Medes & Floyd (1944; Medes, Floyd & Weinhouse, 1946) who demonstrated by means of isotopic labelling that acetate units were the primary product of fatty acid oxidation and ketone bodies were formed from them. Subsequently Lehninger (1945) showed that fatty acid oxidation produced ATP by coupling to oxidative Phosphorylation and that the presence of fumarate in liver preparations oxidising octanoate diverted fatty acid carbon away from ketone body formation in amounts which could be quantitatively accounted for by accumulated citrate, 2-oxoglutarate and succinate (Lehninger, 1946). It had previously been found that acetate and acetoacetate (Rittenberg & Bloch, 1945; Buchanan et al., 1947) were metabolised via the Citric Acid Cycle and later the requirement for a Cycle intermediate to be supplied for complete oxidation of fatty acid to CO_2 and H_2O was noted by Knox, Noyce & Auerbach (1948). The establishment of the mechanism of

β-oxidation, with acetylCoA as the product (Lynen & Ochoa, 1953; Beinert et al., 1953) gave the final evidence for the connection between fatty acid catabolism and the Citric Acid Cycle.

The localisation of the processes of oxidation and respiration within the mitochondria came from work on differential centrifugation of tissue homogenates carried out by Claude, Schneider and co-workers, who localised the succinic oxidase system and the cytochrome c in mitochondria (Claude, 1946; Schneider, 1946; Hogeboom et al., 1946; Schneider et al., 1948). Kennedy and Lehninger (1949) subsequently demonstrated that the mitochondria were the major sites of fatty acid oxidation and Citric Acid Cycle activities within the cell, thus establishing the mitochondria as the 'Intracellular Power Houses' of the cell.

During the 1950's and 1960's research on the Citric Acid Cycle enzymes continued. The individual enzymes were obtained by the ever-improving methods of isolation and purification of proteins, in pure form and detailed studies on the kinetics, reaction mechanism, structure and effectors of each has been carried out. (Chapter II contains a short summary of some of the known properties of each component enzyme of the Cycle.)

Other pathways which feed into the Cycle i.e. whose end products are Cycle intermediates (e.g. breakdown of amino acids), and biosynthetic routes whose starting materials are Cycle intermediates (e.g. porphyrin biosynthesis) have also been much investigated. Such studies led to the full realization of the central role of the Citric Acid Cycle in cellular metabolism, a concept first suggested by Wood (1946), whether it be in a unicellular organism or in a highly specialised tissue such as brain.

The localisation of the Cycle enzymes has also been further investigated. There are a number of Cycle enzyme activities existing extramitochondrially, malate dehydrogenase (see Banaszak & Bradshaw, 1975 for review), aconitase (Eanes & Kun, 1971) and isocitrate dehydrogenase (see Plaut, 1970). The functions of these activities in the cytosol are all thought to be quite distinct from the Citric Acid Cycle e.g. the cytosolic isocitrate dehydrogenase and aconitase are considered to be concerned with the production of citrate from isocitrate and 2-oxoglutarate (derived principally from glutamate) for fatty acid biosynthesis.

The cytoplasmic malate dehydrogenase is generally thought to function in the 'Malate shuttle' for transferring reducing equivalents between the mitochondrial and cytoplasmic compartments of the cell.

Although the presence of these enzymes in the cytoplasm might be said to indicate that a certain amount of part of the Citric Acid Cycle activity is cytoplasmic, it is difficult to see what purpose this would serve unless perhaps the requirement for reducing equivalents in the cytoplasm is much greater than the demand for mitochondrially produced ATP. It is far more feasible that the cytoplasmic species of the enzymes serve quite different functions from the mitochondrial species.

The regulatory features of the enzymes of the Cycle and their possible relevance to the control of the Cycle has also been the topic of much investigation. This will be discussed in detail in Section iii) of this Chapter.

ii) General Mechanisms of Metabolic Regulation

Control can be exerted in a multitude of ways over the whole range of metabolic functions, from the maintenance of homeostasis in the higher organisms to the delicate balancing of metabolite concentrations within metabolic pathways in every cell. Such regulations are due to various types of control mechanism from the highly sophisticated to the very simple. Very many of these have been thoroughly investigated while others have not been assessed or perhaps even discovered yet.

The primary or highest level of control existing in multicellular organisms is that exerted by the hormonal and nervous systems. These are designed specifically to preserve the structural and functional integrity of the entire organism by affording an efficient and sensitive communication network between various organs and tissues. The mechanisms involved in such controls, are highly complex and are still being rigorously investigated.

Although such controls may seem far removed from the control of the Citric Acid Cycle, it should be remembered that an important regulating influence over any pathway is the concentration of the enzymes composing that pathway. These can be governed by a number of factors, the most important of which operate at the level of production i.e. transcription and translation. Hormones may then play an active role in controlling the levels of the Cycle enzymes. Although in the higher organism these concentrations stay remarkably constant (see Srere, 1972) under various metabolic conditions, it has been shown that after regular exercise, the concentrations of some of the Cycle enzymes rise quite markedly (Holloszy et al, 1970). It has also been suggested that the polypeptide hormone, Vasopressin directly interacts with citrate synthase (Horowitz et al., 1966)

though the significance of this in vivo is highly suspect since the concentrations of the hormone required to elicit the inhibition are unphysiologically high (Srere, 1971).

The next level of metabolic control to be considered is the entry and exit of metabolites into and out of the cell. Although this is also within the domain of hormonal and nervous control, other factors, such as the existence and abundance of specialised permease enzymes within the cell membrane, also play an important role. It is well established that insulin controls the rate of entry of glucose into muscle and fat cells (Randle & Morgan, 1962). The flood of Ca^{++} through the sarcolemma of muscle cells prior to contraction is known to be caused by a wave of depolarization (Ebashi & Endo, 1968).

To many normal metabolites the cell membrane poses as an impermeable barrier and the existance of specialised mechanisms for the transport of these substances, in itself may be a regulatory feature. Membrane bound permease enzymes are subject to the same types of control at the genetic and molecular levels as any 'soluble' enzyme.
Those metabolites which can permeate the cell membrane without the aid of a permease are dependent on the process of diffusion.
Although this is perhaps not as controllable a process as enzymic transfer, it is under the constraints of intracellular and extracellular concentrations and the permeability of the membrane to the substrate which is dependent on the composition of the membrane itself.

The study of this type of control of metabolism is still in its primary stages due to the difficulty in working with non-aqueous media although many new techniques (e.g. ESR and NMR probes) are being developed to deal with the study of membrane systems.

The cell membrane can be considered the ultimate site of control of cellular functions but perhaps more important to the control of the Citric Acid Cycle is the transfer of metabolites through the inner mitochondrial membrane. This is a much more impenetrable barrier than the cell membrane, the only metabolites apparently diffusible through this membrane being H_2O, acetate, and CO_2 (Chappell, 1968; Papa & Paradies, 1974). Any other metabolites permeating this membrane do so by specific carrier systems. Antiports (Chappell, 1968) are systems in which the entry of one metabolite only occurs by the exit of another. Many such systems have been found. The movement of the dicarboxylic acids malate, 2-oxoglutarate and citrate (in the dicarboxylic H-citrate^{2-} form) are all indirectly linked to the movement of two hydroxyl ions out of the mitochondria when the phosphate/hydroxyl antiporter is operating, thus these acids distribute themselves according to the ΔpH:

$$\log \frac{\{A^{n-}_{in}\}}{\{A^{n-}_{out}\}} = n\Delta pH$$

(Palmieri et al., 1970; McGiven & Klingenberg, 1971). This has been referred to as the 'cascade' system of carriers. Other established Antiport systems are the glutamate/hydroxyl (Bradford & McGivan, 1973) and the glutamate/aspartate (Tischler & Pachence, 1976) carriers. There is some debate as to whether pyruvate and acetoacetate freely traverse the membrane or are transferred by a specific pyruvate (acetoacetate)/hydroxyl antiporter (Zahlten et al., 1972; Papa & Paradies, 1974; Halestrap & Denton, 1974). The adenine nucleotide translocase is also an antiporter system in that ADP^{3-} enters the mitochondrial matrix only when ATP^{4-} exits.

Since the charge on these two species is not equivalent this is an electrogenic interchanger and responds to the membrane potential ($\Delta\psi$) of the inner mitochondrial membrane (-ve on the inside) hence favouring ADP uptake and ATP expulsion (Buchanan et al., 1976; Vignais, 1976).

A number of important chemical species are known to be impermeant, e.g. oxalacetate, fumarate, CoA, acylCoA esters and pyridine nucleotides. A specialised transporter system involving carnitine exists for the transfer of fatty acids into the mitochondria. The uptake of cations may also be mediated by transfer mechanisms and it has been suggested (Jacobus et al., 1975) that different cations may be transported to varying degrees in the mitochondria of different tissues, indeed, it is true that mitochondria from different tissues may contain different transporter systems e.g. liver and kidney contain an ornithine transporter which is necessary for the Urea Cycle to function (since ornithine transcarbamylase is the only Urea Cycle enzyme contained within the mitochondria).

The existence of such complex and interdependent transporter systems suggests that they may well play a role in the control of mitochondrial function. However as yet there is no conclusive means of quantitatively assessing their role in metabolic regulation, either from the point of view of supply of oxidisable carbon, or the removal of the energy produced as ATP.

Control over cellular metabolism within the confines of the cell membrane can be elicited in a number of ways. Primary control resides at the genetic level i.e. the rate of transcription and translation of the enzymes which together with the rate of catabolism will determine the turnover of these proteins and hence their steady state concentration. Enzyme concentration is a particularly important regulation feature in the in vivo situation since the ability to increase net flux of an enzymic reaction is partly a function of catalyst concentration.

Should an enzyme be operating in the region of its V_{max} then an increase in substrate concentration would have no effect on the throughput of the reaction. The high concentrations of enzymes in cells (Srere, 1968) make this an unlikely possibility and emphasises the importance to metabolism of the control of enzyme turnover. It has been suggested that the turnover time of the Citric Acid Cycle enzymes is short, but the concentrations of these remains remarkably constant within cells (Srere, 1972). It has also been shown that the proportions of the Cycle enzymes to the elements of the respiratory chain is remarkably constant in each tissue of the higher organism (Greville, 1969). This suggests that the synthesis of all of these proteins is linked and must necessarily involve a complex network of controls operating at the genetic level, specific for each tissue.

The turnover of coenzymes is also an important factor to be considered. The absolute amount of these within the cell and particularly within a mitochondrion must again be ultimately controlled by 'genetic' factors. Mitochondrial concentrations of the coenzymes (the pyridine, adenine and guanine nucleotides and coenzyme A) will be of particularly significance to the operation of the Citric Acid Cycle, since these are essentially 'trapped' inside the mitochondrial matrix by the absence of any means of net exit or entry through the inner mitochondrial membrane. Hence ATP can only leave when ADP and phosphate replace it via the adenine nucleotide translocase. NADH can only be reduced by the respiratory chain or the pyridine nucleotide linked dehydrogenases, GDP formed from GTP via the nucleoside diphosphate kinase, and free CoA re-formed by thiolytic cleavage of acylCoA esters. The distribution between the different forms of the coenzymes-because they participate as substrates in many of the reactions of the Cycle - can finely control the operation of Cycle enzymes, thus the absolute concentrations of

the coenzymes is an important feature of mitochondrial metabolism. Thus if say, the mechanisms for the reoxidation of NADH are not functioning then the Citric Acid Cycle cannot operate due to a lack of NAD^+. There is no method of obtaining NAD^+ from any other source. The same is true for the recycling of any of the other coenzymes involved with the Cycle and its related pathways.

One further complication with the pyridine nucleotides is their location within the mitochondria. Although they are unable to traverse the inner mitochondrial membrane, from the fact that mitochondrial content of NAD(H) varies with the type of tissue (as do the cytochromes). Klingenberg (1967) considers these as being implicitly within the membrane space and suggests that the soluble dehydrogenase enzymes are closely associated with, but not contained within, the inner membrane. Equally well the pyridine nucleotides may be located within the matrix and the differences in content between tissues could be due to other factors (the content of so called 'soluble' enzymes also vary from tissue to tissue). Unfortunately there is no way of establishing the placement of the pyridine nucleotides and indeed they may well be distributed between both phases.

The most fully investigated mechanisms of metabolic control are those operating at the enzyme level. These are easily studied since the effects are exhibited by the isolated enzyme. Unfortunately this has also proved a drawback in that the extrapolation of such results to account for <u>in vivo</u> phenomena have often been made too hastily and the physiological significance is suspect (see Srere, 1971). Nevertheless it is true that there are many sophisticated control mechanisms operating at the molecular level which are of great importance to the efficient functioning of metabolism.

One of the most fundamental regulatory mechanisms in biochemistry is that of feedback inhibition. This was proposed by Umbarger as a result of work on biosynthetic pathways in bacteria (Adelberg & Umbarger, 1953; Umbarger, 1956). It was later elaborated into a general regulatory mechanism of intermediary metabolism (Umbarger, 1964). Although this is often referred to as 'end-product' inhibition, it should not be confused with 'product' inhibition which is quite a different phenomenon. Feedback inhibition describes the situation where a metabolite inhibits an enzyme in a pathway which is prior to the enzyme which produces that metabolite. In many cases, particularly biosynthetic pathways, the inhibitory metabolite is the end product of the pathway and the enzyme affected is the initial one in the chain of reactions.

Product inhibition, on the other, is defined as the inhibition of an enzymic reaction by its own product, either by reversal of the reaction or by a specific chemical reaction between enzyme and product at or away from the active site (Walter & Frieden, 1963).

Activation of enzymes also happens by the same types of mechanisms. Feed-forward activation is where a metabolite at or near the beginning of a pathway can activate an enzyme further down the chain (Leloir & Goldenberg, 1960). Activation by the substrates of an enzyme has also been suggested (Kearney, 1957; Hathaway & Atkinson, 1963; Bridger et al., 1968).

These effects are in many cases, due to an alteration in the conformation (3-D structure) of the enzyme protein. Allosterism is the terminology used to describe the situation where metabolites, whether these be substrates, products, coenzymes or other metabolites, bind to the enzyme at sites other than the catalytic centre to produce their effect (activation or inhibition). Many allosteric enzymes exhibit

sigmoidal kinetics as opposed to the hyperbolic binding isotherms of non-allosteric enzymes. Such sigmoid behaviour means that within certain concentration ranges there are large changes in velocity in response to very small changes in substrate concentration hence producing a very sensitive mechanism of control of enzyme activity within such concentration ranges.

In the majority of cases allosteric enzymes are polymeric and consist of more than one type of subunit. There have been two models proposed which explain the phenomenon of allosteric regulation, that of Monod, Wyman & Changeaux (1965) and Koshland, Nemethy & Filmer (1966; Adair, 1925). Both models presuppose the existance of more than one subunit of the enzyme. The Monod-Wyman-Changeaux model proposes that the enzyme exists in at least two different conformational states which differ in their ability to bind the substrate/effector. In presence of low concentrations of substrates (and activators) the enzymes will largely exist in the state which does not effectively bind these (T - state). However as the concentrations of these increase the proportion of the enzyme in the T-state decreases and the substrate binds to the other (R-state) form of the enzyme. Allosteric activators then will increase the proportion of the enzyme in the R-state hence catalysis (initial velocity) is increased with respect to substrate concentration, whereas inhibitors (negative effectors) will increase the amount of enzyme in the noncatalytic T-state hence reducing the catalytic power of the enzyme towards the substrate.

The Koshland-Nemethy-Filmer model proposes that binding of substrate to one subunit will induce conformational changes in that polypeptide which will affect a neighbouring subunit and make this second subunit more amenable to substrate binding. As more substrate binds, binding of further substrate becomes increasingly easy and the sigmoidal binding

isotherm is produced. The presence of activators or inhibitors will affect the conformation of the subunits either by aiding or blocking further conformational changes in neighbouring subunits.

The essential difference between the two models is that the Monod-Wyman-Changeux model proposes that all subunit conformations are identical and the whole enzyme is either in the T-state or the R-state whereas the Koshland-Nemethyl-Filmer model proposes that subunit conformational changes are sequential, binding of substrate to one subunit inducing binding of substrate to the neighbouring subunits and so on. In practice it is very difficult to distinguish the two mechanisms.

Although these models both presuppose the existance of multiple subunits, it has been observed that the monomeric enzyme, hexokinase, is allosterically affected by glucose-6-phosphate (Colowick, 1973). It is also true that very many enzymes exist as polymeric structures, yet only a few display sigmoid kinetics or show any allosteric regulation features.

Changes in the activity of enzymes can be produced by alterations in the quaternary structure. It has been well established that phosphorylation of glycogen phosphorylase brings about an aggregation of less active dimeric to fully active tetrameric form of the enzyme (E.G. Krebs *et al.*, 1966). Also the activating effect of ADP on pig heart isocitrate dehydrogenase is to cause aggregation of monomers into dimers and tetramers (Chen *et al.*, 1964).

That such types of control of enzymes exists is undisputed. However, evaluating their importance to the regulation of metabolism, and indeed establishing *in vivo* enzymic regulation is a much more difficult task. The theory of 'pacemaker' enzymes was first developed by Krebs (1957). He proposed that not all enzymes in a pathway need be

controlled by factors other than enzyme and substrate concentrations, but that one 'pacemaker' at a crucial point would be the most efficient and economical method of control. Teleologically, these would be expected to exist at branch points of metabolic pathways, at the initiation reaction (the first comitted step) of a pathway and at points where energy supply is determined.

Enzymes other than those designated as 'pacemakers' or 'rate-controllers' have rightfully had their importance to metabolism discussed. Such enzymes have often been labelled 'Equilibrium' enzymes, although this terminology is very misleading and incorrect. Should any metabolic reaction be in complete equilibrium this would signify cell death. A much better definition of such reactions would be those whose net flux is very much smaller than the rates of the forward and backward reactions i.e. their capacity to increase net flux is very large. Krebs (1969) pointed out that since most enzymes are not saturated with substrate, then substrate concentrations are very important to rate control, especially with the rate controlling enzymes; the 'equilibrium' reactions must play a key role in the maintenance of and alterations in substrate concentrations. With respect to substrate concentrations, Sols & Marco (1970) drew attention to the fact that great care must be taken when considering the TRUE free concentration of metabolites in the cells. The number of potential binding sites for certain intermediates, particularly coenzymes, is very large and if, as in the case of NADH, binding tends to be very strong then the actual free concentration may be relatively small. Also, many normal metabolites may exist in differing isomeric forms such as keto - enol tautomers, anomeric forms of sugars etc., and generally only one form is specific for the enzyme reaction. Thus the total concentration may not reflect the active concentration as far as the metabolic reaction is concerned. Another point to note

here is the existence of enzymes which catalyse conversion of metabolites to the active form. One such enzyme is oxalacetate tautomerase (Annett & Kosicki, 1969). The existence of such systems makes the calculation of the active form present in the cell, much more complex. In this respect it is also wise to consider in what form of the intermediate is released from the proceeding enzymes which produce it.

iii) Regulatory Features of the Citric Acid Cycle Enzymes.

As discussed in the previous section, external factors such as hormonal or nervous stimuli, and the rate of entry of metabolites into cells, have an indirect function in control over intermediary metabolism. Overriding control of any pathway must be exerted by supply and demand. The question then arises as to how much control is integrated with the pathway; is the flux through the pathway merely dependent on the rate of supply of 'substrate' or the removal of 'product'? Furthermore this leads to the problem of how supply and demand are themselves co-ordinated. Clearly then, the pathway must be considered not only from the point of view of the functioning and control properties of the component enzymes, but also in relation to the entire system in which it is contained.

Sensitive controls at the molecular level must not only function in the balancing of supply and demand but must also participate in the efficient partitioning of metabolites between biosynthetic pathways and energy production. To a certain extent the functions of the Citric Acid Cycle in various tissues are governed by the component enzymes which can have different kinetic features (such as K_m's, V_{max}'s, effector binding) depending on the tissue in which they are found. This fact serves to emphasize that metabolic control does exist at the level of the enzymes of the Cycle, and suggests that control features of the Cycle will vary from cell to cell, or tissue to tissue. In this respect any detailed investigation or discussion of the operation and control of the Citric Acid Cycle in the higher organisms should refer specifically to one tissue. The following discussion will, except when specified,pertain to the operation of the Citric Acid Cycle in heart tissue, since this was the tissue chosen as the basis for the work reported in the following chapters.

The vast majority of oxidizable carbon is supplied to the Cycle as acetylCoA, no matter which tissue or organism one considers (only in extreme dietary conditions where protein is being broken down are Cycle intermediates produced in significant quantities to become a major supply of oxidizable carbon). However the ultimate source of the acetylCoA - fatty acids, glucose or ketone bodies - will generally be dependent on the dietary state; moreover, hormonal mechanisms will control blood levels of these precursors. Although various tissues do exhibit preference for different precursors, this is generally accepted as being a function of hormonal influence operating at the outer cell membrane. It is well established that brain tissue almost exclusively oxidizesglucose (Cahill & Owen, 1968) and that heart muscle preferentially oxidizes ketone bodies if they are available (Barnes et al., 1938; Hall, 1961; Williamson & Krebs, 1961; Olsen, 1962; Little et al., 1970) but in general most tissues will oxidize whatever is available and this is also true for heart tissue.

The control of 'supply' to the Citric Acid Cycle in any tissue could be considered to be located at many points, the entry of oxidizable substrate into the cell, the flux through glycolysis or the rate of fatty acid breakdown. The controls on these processes have accordingly been much investigated. The pyruvate dehydrogenase complex can be considered as one immediate source of substrate acetylCoA for the Cycle. It is located within the mitochondrial matrix and hence must to some extent be dependent on the rate of entry of pyruvate through the mitochondrial membrane. As mentioned previously the mode of entry of

pyruvate is still a subject of debate. However pyruvate dehydrogenase itself exhibits many regulatory features. The enzyme consists of a complex arrangement of three types of subunit (Reed & Cox, 1966) and is subject to control by substrate and product availability, but although the ratios of acetylCoA: free CoA and NADH:NAD affect the activity of the isolated enzyme it is difficult to show these are major controls *in vivo* (Lowenstein, 1970).

The enzyme is also subject to regulation by phosphorylation and dephosphorylation catalysed by specific kinase and phosphatase enzymes associated with the complex (Linn, Pettit & Reed, 1969). Phosphorylation deactivates the enzyme and this has its effect entirely through the decarboxylase subunit, as neither of the other two subunit types are phosphorylated. Reed *et al.* (1974) demonstrated that operation of the kinase is dependent on $MgATP^{2-}$ concentration and the phosphatase is dependent on free Mg^{++} and Ca^{++} concentrations. These workers suggest that control is exerted principally by the ATP:ADP ratio which will in part determine the free Mg^{++} and Ca^{++} levels in the mitochondrion. Pyruvate has an inhibitory effect on the kinase and may also be an important regulator of activity of pyruvate dehydrogenase.

The necessity for the strict control of pyruvate dehydrogenase arises from the fact that β-oxidation of fatty acids produces acetylCoA and under conditions of fat breakdown there is a need for retarding pyruvate oxidation both from the aspects of overproduction of acetylCoA and of enhancement of gluconeogenesis. In perfused hearts it has been demonstrated that during fatty acid breakdown, pyruvate dehydrogenase is inhibited. Lowenstein (1970) has proposed that this is an inhibition of pyruvate dehydrogenase by acetylCoA.

Recent work on the *E.coli* pyruvate dehydrogenase (Shepherd & Hammes, 1976) has indicated that acetylCoA shows positive homotropic

allosteric effects, although the relevance of this to metabolism and the existence of the phenomenon in the mammalian enzyme has yet to be established.

The oxidation of fatty acids also takes place within the mitochondria and relies on efficient transfer of fatty acids through the inner mitochondrial membrane by the carnitine acyl transferases. Because the process of β-oxidation entails the action of a series of enzymes, the control over the pathway is complex. The major factors prevailing are the availability of carnitine and CoA, the efficient oxidation of NADH and $FADH_2$ to provide the necessary NAD^+ and FAD, and an efficient means of utilizing the acetylCoA produced.

The ketone bodies, acetoacetate and β-hydroxy butyrate, are a very important source of acetylCoA, especially in heart muscle. They are produced in the liver from acetylCoA via β-hydroxy β-methyl glutarylCoA which is cleaved to form acetoacetate and acetylCoA. The acetoacetate and its reduced form, β-hydroxy butyrate, are released into the bloodstream where an important organ which removes them is the heart. Once into the tissues the β-hydroxy butyrate is oxidised to acetoacetate via the action of β-hydroxy butyrate dehydrogenase - a mitochondrial enzyme (Wakil, 1963). Acetoacetate is broken down into two acetylCoA units by the actions of the enzymes 3-keto acid CoA transferase and β-keto thiolase (acetylCoA acetyl transferase) (Greville & Tubbs, 1968). The physiological controls on these enzymes have not been investigated, but regulation may be accomplished <u>in vivo</u> by substrate availability, especially the levels of succinylCoA, acetoacetate and free CoA. One aspect of ketone body catabolism which has not been investigated is the relationship between the enzymes succinyl thiokinase and 3-keto acid CoA transferase. In tissues which are metabolising acetoacetate the flux through succinyl thiokinase

must be severely reduced, since much of the succinylCoA formed by the 2-oxoglutarate dehydrogenase is used to form acetoacetyl CoA by the transferase enzyme. This implies that either the transferase has much more affinity for succinylCoA than does succinyl thiokinase, or that the activity of succinyl thiokinase is in some way retarded. It has been suggested that the major control over succinyl thiokinase is exerted by the ratio of GTP:GDP (LaNoue et al., 1972) and that this ratio is in equilibrium with the mitochondrial ATP:ADP ratio, although in liver mitochondria these ratios do not appear to be in equilibrium (Bryla et al., 1973). The question then arises as to how the operation of these enzymes is integrated and what the effect on Cycle operation will be during acetoacetate catabolism.

Degradation of amino acids produces both acetylCoA (and its precursors) and Cycle intermediates. Control over these pathways appears to have been little investigated particularly in relation to their effect on the Citric Acid Cycle. Exceptions to this are glutamate and aspartate. These act as important anaplerotic sources of Cycle intermediates (LaNoue et al., 1970) and have been implicated in a specialised 'shuttle' mechanism for the movement of reducing equivalents between the cytosol and mitochondrion (Borst, 1963; Williamson et al., 1973; LaNoue et al., 1974).

The control over the Citric Acid Cycle demand for energy production or intermediates for biosynthetic pathways is much more intricate and less well defined. The Cycle may have an anaplerotic function or a purely oxidative function, depending on the tissue concerned. For instance in heart muscle the function of the Cycle is almost purely oxidative i.e. it can be regarded as exactly balanced (acetate in = CO_2 out), whereas in liver the function of the Cycle is predominantly anaplerotic. Since the energy produced by the Cycle

through the Electron Transport Chain in the liver cell is used in biosynthetic pathways, and most of the metabolites produced are exported, the flux through the Cycle in liver will not be so strictly governed by the energy demand of the tissue itself. The energy produced can be channelled off into say, production of cholesterol or of fatty acid, which can then be disposed of by transport out of the cell to the body's fat depots. Quite the opposite situation exists in heart muscle where the energy liberated (ATP produced) must be very closely related to the demand for energy for muscle contraction within the heart tissue itself. There is very little, if any, diversion of excess energy into biosynthetic routes.

Investigations into the control of the Cycle had been preceded by considerations of control of oxidation in relation to glycolysis. A main factor was shown to be the availability of intracellular orthophosphate and phosphate acceptors (Lardy & Wellman, 1952). Chance and Williams (1956) also outlined at length the importance of the availability of ADP to metabolic regulation.

These ideas were later extended by Krebs (1959) when he showed that respiration was also dependent on NAD^+ concentration. Soon afterwards Krebs turned this reasoning to a consideration of the control of the pathways which feed electrons into the respiratory chain (Krebs & Lowenstein, 1960). The major conclusion was that the Citric Acid Cycle is controlled at the citrate synthase reaction and although it was not clear how this regulation was accomplished, Krebs suggested that the concentration of oxalacetate, acetylCoA or citrate synthase, or a combination of these was the most likely.

Much ensuing research was devoted to finding a mechanism which would control the activity of citrate synthase. One of the first regulators to be suggested was ATP. This inhibits the reaction by

competition with acetylCoA for the active site (Hathaway & Atkinson, 1965; Shepherd & Garland, 1966; Kosicki & Lee, 1966). Williamson et al. (1969) could not observe this effect and it has since been suggested that it was unphysiological since $MgATP^{2-}$ (the prevalent form in mitochondria) is not inhibitory (Srere, 1971). Williamson's group demonstrated a correlation between the flux through citrate synthase and the redox potential of the pyridine nucleotides which is highly suggestive of the regulation of citrate synthase by the availability of oxalacetate. If this is so, regulation by the adenine nucleotides would be indirect, exerted through their effect on the respiratory chain. Subsequently this group proposed that succinylCoA could inhibit citrate synthase by competition with acetylCoA (Smith & Williamson, 1971; LaNoue et al., 1972), and on the basis of experiments with heart mitochondria suggested that this effect was physiologically significant under certain conditions.

Srere and Matsuoka (1972) have demonstrated that in vitro citrate synthase is inhibited by NADH and acetoacetylCoA, both of which are competitive with acetylCoA, but the physiological significance of these inhibitions has not been assessed. The enzyme is also sensitive to variations in ionic strength (Wu & Yang, 1970), the amount of free Mg^{++} (Kosicki & Lee, 1966) and the pH (Eggerer et al., 1964).

Isocitrate dehydrogenase has also been considered as a major control point of the Citric Acid Cycle, as a result of investigations on the complex allosteric effects exerted on the NAD^{+} linked enzyme in vitro by the adenine nucleotides, Cycle intermediates and some cations (Hathaway & Atkinson, 1963; Atkinson, 1969b; Plaut, 1970). This proposition has also been made from a teleological point of view, since citrate (or isocitrate) must be exported from the mitochondria before fat synthesis can take place (Srere, 1968), and under these

conditions a control point in the Cycle after the citrate synthase step would be desirable. However this would not be important in heart metabolism since heart does not synthesize appreciable quantities of fatty acids. The existence of two forms of isocitrate dehydrogenase, the NAD^+- and the $NADP^+$-linked enzymes, has also been the subject of much discussion. Why should both exist? Which plays the major role in the Citric Acid Cycle? The two species of the enzymes differ in size and properties (Plaut, 1970). In higher organisms the NAD^+-linked enzyme is confined to the mitochondria whereas the $NADP^+$-linked enzyme is distributed in both mitochondrial and cytoplasmic compartments of the cell. The ratio of the two mitochondrial enzymes varies between different tissues (Stein et al., 1967). In heart mitochondria the $NADP^+$-linked enzyme is more active than the NAD^+-linked enzyme, and for this reason it has been proposed that the former would predominate on the Cycle (Stein et al., 1967). However, on the basis that the NAD^+-linked isocitrate dehydrogenase, cytochrome a and malate dehydrogenase form a very nearly constant proportion group in all tissues, Goebell & Klingenberg (1964) suggest the NAD^+-linked isocitrate dehydrogenase is that which functions as part of the Citric Acid Cycle. Nicholls & Garland (1969) also agreed with this proposal since they found that in liver mitochondria the rate of transhydrogenation between NADPH and NAD^+ was too slow to cope with the flux through the Cycle under normal conditions.

One reason for the existence of the two forms of the enzyme in the mitochondria has recently been suggested by Colman (1975). She has shown that the ionic form of isocitrate which is the substrate is different for each enzyme, the NAD^+-linked enzyme being specific for dibasic isocitrate, the $NADP^+$-linked enzyme for tribasic isocitrate. Since the distribution of these two forms of isocitrate in the mitochondria

will depend on pH, the nature of the unbound metal ions present and the concentration of chelators, variations in these factors will cause changes in the proportion of isocitrate metabolised by each enzyme. The significance of this is thought to be in adjusting the ratio of NADH:NADPH within the mitochondria; thus both enzymes may participate in the Citric Acid Cycle and so play a complementary role in metabolism. It would not be unreasonable to suggest that conversion of isocitrate to 2-oxoglutarate within the mitochondrial matrix is not exclusively the domain of either the NAD^+- or the $NADP^+$-linked isocitrate dehydrogenase. However, inclusion of both enzymes in a consideration of the control and functioning of the Cycle would then involve additional constraints, such as the ratio of $NADP^+$:NADPH and the rate of transhydrogenation within the mitochondrial matrix. As rather little is known about these constraints, it was decided for the purposes of this study to consider only the NAD^+-linked enzyme as participating in the Citric Acid Cycle. This decision did not in any way alter the conclusions drawn from the results, as will be seen in Chapters IV and V.

Study of the other enzymes of the Cycle reveals that not one is without a proposed regulation mechanism, but these are generally regarded as unimportant to the control of the Cycle *in vivo*. The effects of various metabolites on succinic dehydrogenase have been known for many years (Kearney, 1957) but it has been shown that the catalytic capacity of the enzyme *in vivo* never falls below the cycle flux (Singer *et al.*, 1973). The inhibitory effect of oxaloacetate on succinic dehydrogenase is well known (e.g. Zeilemaker, Klaase & Slater, 1969), although Gutfreund & Jones (1964) suggested that the succinic dehydrogenase is inaccessible to oxaloacetate in the physiological situation. A more recent report (Ackrell, Kearney &

Mayr, 1974) has indicated that oxaloacetate does play a role in the deactivation-activation 'cycle' of succinic dehydrogenase, since oxaloacetate will bind to the deactivated enzyme and is released on activation. The significance of this to the operation of the Citric Acid Cycle is probably negligible since even the deactivated enzyme in vivo does not become rate-limiting for the Cycle flux. It may be that the deactivation-activation properties of succinic dehydrogenase play a role in the efficient channelling of electrons through the electron transport chain (Gutman et al., 1971b; Singer et al, 1973).

The binding of oxaloacetate to succinic dehydrogenase has been suggested to be of importance to the Cycle regulation from another point of view, that of decreasing the mitochondrial concentration of oxaloacetate (Sols & Marco, 1970). The implications of this will be more fully discussed later (see Chapter IV).

The inhibition of fumarase by ATP (Penner & Cohen, 1969) has also been dismissed as irrelevant in mitochondria in vivo (Krebs, 1970). As with the case of citrate synthase, MgATP does not inhibit.

iv) Control of the Citric Acid Cycle as a whole

The basic objection to many proposed regulations of the Citric Acid Cycle has been that they were extrapolated from work on the isolated enzymes. The real point of research into control mechanisms is to understand what regulates metabolism as a complete entity. Although the study of the individual enzymes is necessary to find out possible control mechanisms, the results must be viewed in the context of the cellular situation.

This was the point that Srere was making when he said "what enzymes of the (Citric Acid) Cycle are not controlled? Probably none. ..... the sooner we view regulation as a pervasive factor in metabolism operating at every enzyme step, the sooner experiments can be designed

to challenge and test the hypothesis of 'redundant' multivariate control mechanisms in metabolic regulation".

In agreement with Srere (1971), Rolleston (1972) and Kacser & Burns (1973) point out that control is not necessarily a feature that can be put down to any one part of a metabolic pathway, but is a function prevading the entire system and must be highly integrated between the different pathways for economical and efficient metabolism to exist. Atkinson (1966, 1971) considered that in the cytosol this was due to the coupling of metabolic functions through the adenine nucleotides, because control by the levels of ATP, ADP and AMP is universal throughout the cytosol.

Recent studies on the control of the Citric Acid Cycle have tended to be more realistic about physiologically significant control and the use of intact mitochondria or complete tissues is becoming increasingly the preferred mode of investigation.

Using perfused liver, Krebs (1970) concluded that the concentrations of ATP and oxaloacetate were instrumental in regulating the activity of citrate synthase during ketogenesis and that control on the various routes for the production of acetylCoA were important.

LaNoue, Nicklas and Williamson (1970) used rat heart mitochondria to examine the control of the Citric Acid Cycle. They suggested that since the pathway is strongly linked to the cytochrome chain via NADH and reduced flavoprotein, overriding control may be imposed by the coupling of oxidative phosphorylation to the electron transport chain, which will be dependent on ADP availability. 'Feedback' from the respiratory chain would operate on pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase, and they concluded that the concentration of oxaloacetate will be of key importance to the flux through the Cycle.

Working with rat heart perfused with acetate, Randle, England & Denton (1970) concluded that the Citric Acid Cycle operates in two spans: acetylCoA to 2-oxoglutarate, and 2-oxoglutarate to oxaloacetate, which are controlled at citrate synthase and 2-oxoglutarate dehydrogenase respectively, although other control points could not be ruled out. They noted that acetate utilization suppressed glucose oxidation, increased the concentrations of citrate, isocitrate, 2-oxoglutarate and glutamate, and decreased the concentration of aspartate and free CoA.

A later study by Williamson's group (LaNoue et al., 1972) suggested that the activity of citrate synthase is dependent on oxaloacetate and acetylCoA levels, and that under certain circumstances (high oxaloacetate and low acetylCoA) succinylCoA could be inhibitory both towards citrate synthase and 2-oxoglutarate dehydrogenase. They also suggested that pyruvate oxidation is not rate-limiting for the Cycle, and when pyruvate is the major or sole substrate, pyruvate dehydrogenase is controlled by the rate of utilization of acetylCoA; when fatty acids or ketones are supplying acetylCoA, on the other hand, the phosphorylation state of pyruvate dehydrogenase will be the predominant control factor. Their major premiss is that the adenine nucleotides control the Cycle indirectly through their effects on the concentrations of oxaloacetate, acetylCoA and succinylCoA.

These studies all basically agree that the fundamental controls over the operation of the Cycle in vivo in heart tissue are the redox state of the pyridine nucleotides and the phosphorylation state of the adenine nucleotides, i.e. control by demand for energy. Although the various studies have suggested how these fundamental controls might interact with the Cycle enzymes to regulate Cycle flux, there is no general agreement as to how control is correlated at the level of the Cycle enzymes. If Srere's view is correct, all the enzymes will contribute

to Cycle regulation to a greater or lesser extent depending on the conditions under which the Cycle is operating, and the reason for the differing conclusions made from each study would be a consequence of the varying conditions under which the measurements were made.

Control of the Citric Acid Cycle is still very much an open question, and its operation as an integrated system warrants much more investigation. One way in which such an investigation can be undertaken is by computer simulation. Simulation is defined as the construction and use of models to aid the evaluation of ideas and the study of dynamic systems or situations. It is now a recognised technique for the study of biochemical phenomena (Garfinkel et al., 1970). Very many simulations of complex biochemical systems have been constructed, particularly of the glycolytic pathway (Chance et al., 1960; Garfinkel & Hess, 1964; Martiny, 1972), although many other complex models have been constructed e.g. the respiratory chain (Chance, 1967; Wagner et al., 1971) and perfused heart metabolism (Garfinkel et al., 1974). Few simulation studies have been carried out on the Citric Acid Cycle. A number of simulation studies have included a representation of the Cycle as part of a much larger system, e.g. those of Van der Berg & Garfinkel (1971), Haut et al. (1974) and Garfinkel et al. (1974). Only one simulation of the Cycle as such has been performed (Garfinkel, Williamson & Olsen, 1969). It was based on experimental measurements made with perfused rat liver and was composed of a series of reactions representing the rates of change of one metabolite to another (enzymes were not included in the model). The model simulated the experimental results very closely; the conclusions reached were that the major factor in the regulation of the Citric Acid Cycle is the ratio of NAD^+:NADH, with the activity of citrate synthase being of somewhat lesser importance but nevertheless a significant control feature.

The state of knowledge of the reactions composing the Citric Acid Cycle and the facilities for performing simulations (see Chapter II) have reached such an advanced stage that simulation of the Cycle, involving all that is known about the component enzymes and their reactions, has become a feasible approach to the problem of the Citric Acid Cycle regulation.

This approach i.e. where the model is constructed 'theoretically' from the known properties of the enzymes is one that has not hitherto been tested. All previous models incorporated only the rates of conversion of the metabolites, which were adjusted until the levels of intermediates, the Cycle flux, etc. agreed with the experimental observations (e.g. Garfinkel et al., 1969).

An advantage of simulating the Cycle is that its operation as an intact system can be examined. Systems analysis is a method of study which is fairly new to the life sciences but is rapidly gaining attention (Zeigler & Weinberg, 1970; Guyton et al., 1972; Stark, 1973). It is a basic principle of systems analysis that the properties of a system reflect those of all the individual components as well as showing properties peculiar to the intact system. One example of this in biochemistry is the oscillatory behaviour of glycolysis in yeast under certain conditions (Hess & Boiteaux, 1971); oscillations cannot be observed at the enzyme level, they are a feature unique to the intact system. By using a computer simulation model of the Citric Acid Cycle it is possible to evaluate at least some of the 'system' properties i.e. interrelationships between enzymes, coenzymes or substrates within the Cycle which would not or could not be observed by an examination of the properties of the individual enzymes. There is also the very large advantage with a simulation of this sort, that the changes occurring in all the metabolites present

(including enzyme bound intermediates) can be 'seen' as the conditions are varied, not as in the case of experimental studies, those that the researcher chooses to analyse. There is no need to decide beforehand which intermediates to measure.

Perhaps the major advantage of using a simulation model to investigate the control properties of the Cycle as opposed to using such experimental systems as perfused organs or mitochondrial suspensions, is that the Cycle can be studied in isolation. This means that such complications as cytoplasmic metabolism or transport across the inner mitochondrial membrane need not be taken into account. Such a study can then be enlarged in a step-wise fashion so that the relationships between the Cycle and its adjoining pathways can be evaluated without having to have all the pathways present together, as they would be in a real situation. In this way, understanding of the control of a large area of metabolism can be built up bit by bit, instead of having to rationalise the gross effects exhibited by an in vivo system in terms of the very many pathways which may contribute to these effects.

Before any investigation of metabolic regulation can be undertaken, the criteria for assessing which reactions are exerting control must be defined. The criteria that have been used for the studies reported here are those proposed by Kacser & Burns (1973; c.f. Higgins, 1965[a]; Heinreich & Rapaport, 1974). These authors have suggested a means of comparing the importance of one enzyme against another by the assignment of coefficients, which are calculated from experimental values, to each enzyme. Their theory attempts to put on a logically sound basis the definitions, concepts and criteria of control. Many of the experimental measurements that would be required for a full analysis of a system by the method of Kacser & Burns are as yet impossible to perform, but the technique does lend itself extremely well to the type

of study that can be performed on a computer simulation model. Its use is described in detail in Chapter III.

One final justification for the use of a computer simulation model to study the regulation of the Citric Acid Cycle is that put by Martin (1968): "We should apply computer simulation whenever the mere process of constructing a simulation of a system can in itself be a beneficial learning experience of the system processes. This process can provide an experience that cannot be gained in any other way".

CHAPTER II

CONSTRUCTION OF THE SIMULATION MODEL OF THE CITRIC ACID CYCLE

i) Introduction

Programming for a computer requires that the problem must be expressed mathematically, or at least must lend itself to a mathematical method of solution. Simulation is just such a process since this involves examination of the results of changing one or more of the variables in the system over a period of time, i.e. the system can be represented as a set of time-dependent differential equations and the values of the variables at any point can be established by integration of these differential equations.

Most of the biochemical simulation work before about 1960 was limited to analogue computers, as these were then the most practical way of dealing with such systems. They are extremely useful for simulation, since the variables are easy to manipulate and simulation of the system under a variety of conditions is easily executed. They heralded the introduction of the computer as a very useful tool for the investigation of many complex biochemical problems. The use of analogue computers for simulation is however not entirely satisfactory for two major reasons. Firstly they have a limited capacity, and can cope with only relatively few variables at a time; secondly they cannot handle non-linear equations very well, since multiplying variables together may produce voltages that are difficult or impossible for the machine to cope with. Although some advances were made in the constructing of apparatus to deal with these problems (Chance et al., 1951; Higgins, 1965a) there were still limitations to the systems that could be handled (Garfinkel, 1969). Even a simple biochemical system such as a one-substrate enzyme reaction generates non-linear equations involving the variables E and S,

and more complex enzymes or systems produce equations in even more variables. Consequently, biochemical systems simulated by analogue computer were either relatively simple or not treated to their full complexity (Estabrook, 1962; Higgins, 1964; Morales & McKay, 1967).

The transition to simulation by digital computer was a gradual process. Up until the late 1960's the most popular methods used for digital simulations were a) Turing machines and b) Stochastic methods, neither of which of were entirely satisfactory but could be used to study quantitative behaviour of a system (Garfinkel, 1966). The most direct approach to the simulation of enzyme reactions is through the use of the appropriate differential equations, but these could not then be used due to the problems of integrating them with respect to time.

Numerical integration of differential equations is a method of approximating their values. The accuracy of the approximation is dependent on the step length between the successive integrations and on the rates of change of the variables. The use of a digital computer enables the process to be performed much faster than manual methods and it also allows the process to be carried out on many variables simultaneously. The non-linear differential equations representing biochemical systems involve interacting variables i.e. they contain terms in which two variables, say S_1 and S_2, must be multiplied together. It is essential in such situations that the integrations on the different variables be carried out simultaneously.

This is not an appropriate place to discuss extensively the factors which govern the choice of numerical integration methods. A simple analysis with reference to biochemical problems has been given by Hemker (1972). Here one need only say that since numerical

integration produces only an approximation to the value of a variable at time t, given the value at time 0 and an equation which is explicit in y, the fundamental problem is to ensure that the approximation is accurate for all values of t. Inaccuracy can be introduced by rounding errors, which arise from the rounding upwards or downwards which is necessary to provide the last digit which the computer storage can hold for a single variable, and truncation errors. The latter are more serious. They arise from the fact that the new value of the variable $y(t+\delta t)$ is estimated from the old value $y(t)$ by the use of a series formula involving progressive powers of a step length h (Δt, where $h < 1$). This is true for all numerical integration methods.

For practical purposes the evaluation of this series must be cut short, and the error introduced by the neglect of that part of the series which was not computed is the truncation error. The user desires to keep this error within known limits. Numerical analysis refers to the order, p, of the approximation formula which means that when the series is truncated at the term involving h^p, the error lies between 0 and h^{p+1}. In principle, both h and p can be varied within a single computation.

If the error limits which one specifies are absolute, h^{p+1} must clearly be very small to accommodate the variable of smallest numerical size, which means that calculations of high order must be performed at many steps. The specification of relative accuracy, say 1% for each variable in the system, is therefore preferable.

It is also necessary that the numerical method used should be stable for the differential equations to be integrated. In mathematical terms, stability means that any errors introduced, for example by truncation, should die away, and not systematically accumulate.

Hemker (1972) gives an example of unstable integration using the old-fashioned Euler method. Unfortunately biochemical systems tend to produce sets of differential equations that are 'stiff', that is, they may have rapidly varying values at early times, but converge to slowly varying values at large times, no matter what the initial conditions. It has long been known that well-established methods for integration tend not to be stable for stiff equations unless the step length is very small, which makes the compututation very slow, even with a digital computer.

The predictor-corrector method proposed by Gear (1971) and incorporated by Chance & Curtis (1970) into simulation package, can be shown to be stable for stiff equations, and it also allows the order and step length to be rapidly decreased and increased, respectively, as the transients die away. It has made the numerical integration of systems combining up to 100 variables (as in the Citric Acid Cycle) a practical proposition.

The Chance-Curtis (1970) package also included a machine-independent chemically orientated language for the input of information (Garfinkel, 1966; Chance & Shephard, 1969) and brought the process of simulating enzymic reactions and systems of enzymes on to a more realistic and easily comprehensible level.

Another time-consuming factor involved in the simulation of biochemical systems is the handling of sparse matrices. These arise because each of the variables usually only appears in a few of the reactions, hence a matrix corresponding to the multiplier terms for each of the rate constants contains many zero terms (i.e. in a matrix of rate constants versus variables, many of the positions will be vacant). Curtis & Reid (1971) developed a method for dealing with such sparse matrices, and this along with other refinements were

incorporated into the original version of the simulator program (Curtis & Chance 1972). The new version of the program uses much less computer time for simulation, and can cope with larger systems. One drawback is that the program requires a large amount of computer space (130 Kbytes) and is only suitable for use on relatively large machines. The program is called CHEK (short for chemical kinetics) (Curtis & Chance, 1974) and is available for use on suitably large installations such as the ICL 4-75 machine situated in the Edinburgh Regional Computing Centre (also suitable for IBM 370 or 360 installations).

An advanced version of CHEK, referred to as FACSIMILE, which is a more versatile program as far as input and output requirements and type of simulations performed, has now been produced (Curtis, 1976).

The development of the CHEK program is a major breakthrough in the field of biochemical simulation, in that the mathematical and computational problems have been solved and it allows the simulation of systems hitherto considered too complex to be studied in this way.

ii) General Methods

Simulation of enzymic reactions using the CHEK program, requires expressing the reaction as a set of equations such as those shown, in Fig. II.1, for a simple enzyme mechanism.

To set up a simulation of the cycle the enzymic mechanism and the rate constants for each equation in the mechanism, for all of the enzymes involved in the cycle, are required. If the mechanism of an enzyme has been established, the rate constants for this can be obtained from kinetic measurements. The procedure for evaluating the rate constants is illustrated by the example of the Ordered Sequential Bi-Bi reaction mechanism, the rate equation for which is shown in Fig. II.2. From this equation the kinetic constants can be expressed in rate constant terms (see Cleland, 1963), as in Fig. II.3.

Fig. II.1. Simple Enzyme Mechanism expressed as Chemical Equations and Differential Equations denoting the Rates of Change of the Variables.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E.S.$$

$$E.S. \xrightarrow{k_3} E + P$$

$$\frac{d[S]}{dt} = k_2 \{E.S\} - k_1 \{E\} \{S\}$$

$$\frac{d[E]}{dt} = (k_2 + k_3) \{E.S\} - k_1 \{E\} \{S\}$$

$$\frac{d[P]}{dt} = k_3 \{E.S\}$$

Fig. II.2. Schematic Representation of the Mechanism and the Corresponding Rate Equation for an Ordered Sequential Bi Bi Reaction.

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E.A$$

$$E.A + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (E.A.B)(E.P.Q)$$

$$E.P.Q \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E.Q + P$$

$$E.Q \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + Q$$

$$v = \frac{(k_1k_3k_5k_7A.B - k_2k_4k_6k_8\ P.Q)\ Et}{k_2k_7(k_4+k_5) + k_1k_7(k_4+k_5)A + k_2k_8(k_4+k_5)Q + k_3k_5k_7B + k_2k_4k_6P + k_1k_3(k_5+k_7)A.B. + k_6k_8(k_2+k_4)P.Q + k_1k_4k_6A.P + k_1k_3k_6A.B.P + k_3k_5k_8B.Q + k_3k_6k_8\ B.P.Q}$$

Fig. II.3. Ordered Sequential Bi Bi Reaction Mechanism - Kinetic Parameters expressed in Rate Constant Form.

$$V_{max}^{forward} = \frac{k_5 k_7}{(k_5+k_7)} \qquad V_{max}^{backward} = \frac{k_2 k_4}{(k_2+k_4)}$$

$$K_m^A = \frac{k_5 k_7}{k_1(k_5+k_7)} \qquad K_m^P = \frac{k_2(k_4+k_5)}{k_4(k_2+k_4)}$$

$$K_m^B = \frac{k_7(k_4+k_5)}{k_3(k_5+k_7)} \qquad K_m^Q = \frac{k_2 4_4}{k_8(k_2+k_4)}$$

$$K_{eq} = \frac{k_1 k_3 k_5 k_7}{k_2 k_4 k_6 k_8}$$

Can also be defined (Cleland (1963))

$$K_i^A = \frac{k_2}{k_1} \qquad K_i^B = \frac{k_2+k_4}{k_3}$$

$$K_i^P = \frac{k_5+k_7}{k_6} \qquad K_i^Q = \frac{k_7}{k_8}$$

Rate constants, A, B, P and Q as in Fig. II.2.

If values for all the kinetic parameters are known the values for each of the rate constants can be obtained by solving these identities as a set of simultaneous equations.

Where the mechanism of an enzymic reaction has not been definitely established, the information available can be used to construct a skeleton mechanism which can be used as the best approximation. It is more common with simulation studies to find that a complete set of kinetic constants for an enzyme is not available. In such a situation, precise values of all the rate constants cannot be found, however, estimates of the unknown rate constants can be obtained from the known kinetic relationships, using an optimization technique. The procedure used in this study is called SIMPLEX (Nelder & Mead, 1965; Davis & Ottaway, 1972). It is a statistical technique for the estimation of parameters which will be more extensively discussed in Chapter III. When this technique is applied in order to obtain estimated rate constants, it is often the case that a 'global minimum' i.e. a set of rate constants which fits the data uniquely better than any other set, is **not** found or cannot be conclusively identified, and a range of sets of rate constants may be produced which will yield the values of the known kinetic constants to within a certain limit of error. For purposes of simulation the choice of the rate constants is restricted to a set which corresponds to the values of those kinetic constants which have been measured, and yields reasonable and realistic values for the unknown kinetic parameters. The criterion that all rate constants must be above zero was also employed. On a few occasions, the situation arose where only a multiple of two or three constants could be assigned, hence these values could theoretically be set anywhere between infinite limits (e.g. if, say, $k_9 k_{11} k_{13} = 10^5$,

It should be pointed out here that the conditions of pH, ionic strength and temperature, under which the kinetic parameters used to construct the simulation models of the enzymes were measured, were not standard; various conditions were used in the different studies.

then k_9 could be set at 10^{-10}, k_{11} at 10^{10} and k_{13} at 10^5). In such cases constants were given realistic values, which could usually be of the same order of magnitude (in the example above say 40,50 and 50 for the three constants).

Each enzyme to be simulated was considered separately. A literature survey was carried out to obtain the available details of its mechanism, kinetics, inhibitors and activity. If it was necessary to calculate the rate constants by means of SIMPLEX, a simulation of that enzyme alone was set up and run with the CHEK program to locate any possible errors. In the next section, the details of each of the component enzymes of the Citric Acid Cycle are discussed, in the light of the available experimental information.

iii) Simulation of the Component Enzymes of the Citric Acid Cycle

Citrate Synthase (EC. 4.1.3.7)

This enzyme catalyses the overall reaction:

$$\text{acetylCoA} + \text{oxalacetate} + H_2O \rightleftharpoons \text{citrate} + \text{CoA} + H^+$$

In eukaryotic cells it is found only in the mitochondrial matrix and in glyoxisomes (Brenner & Ames, 1971; Franklin & Luria, 1961; Gilbert & Müller-Hill, 1966). The pig heart enzyme has a molecular weight of approximately 10^5 daltons; it consists of two identical subunits, each with one active site (Singh et al., 1970; Wu & Yang, 1970).

The mechanism proposed for the enzyme is Rapid Equilibrium Random Bi Si Sequential (Shepherd & Garland, 1969; Srere, 1972), although Johansson et al. (1973) report that an ordered mechanism with oxalacetate binding first is not entirely ruled out. It has been shown (Smith & Williamson, 1971; Srere, 1972) that citrate competitively inhibits oxalacetate binding and CoA is also a competitive inhibitor of acetylCoA. As the forward and backward

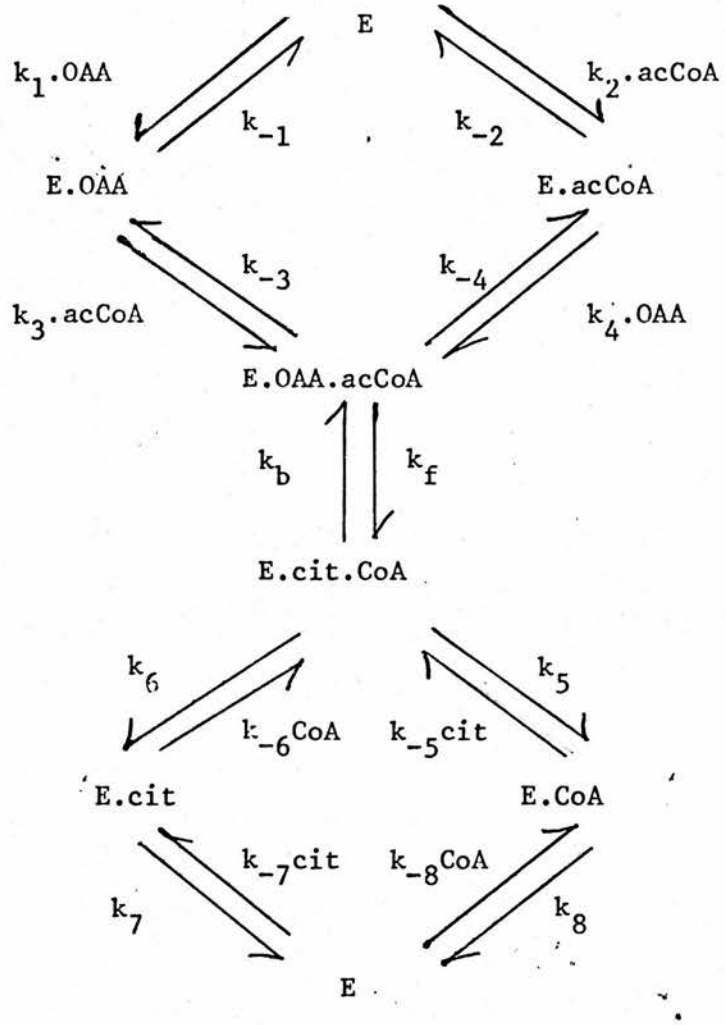
reactions have different pH optima, Srere (1972) has suggested that there are two ternary complexes involved, and that once both substrates are bound to the enzyme, a proton is removed from the methyl group of the acetylCoA and that the carbanion so formed attacks the keto carbon of oxalacetate to form a lactone intermediate. The attack of the carbanion on the keto carbon is suggested by Bové et al. (1959) to be the rate limiting step.

The mechanism adopted for the simulation was Rapid Equilibrium Random Bi Bi - that is, the catalytic step between the two ternary complexes is rate limiting (Fig. II.4). The kinetic parameters used for evaluating rate constants were those relating to the enzyme from rat tissues (Srere, 1972). The K_{eq} was taken as 4.65×10^5 (Stern et al., 1952) and the Turnover Number 17,000 min^{-1} (Singh et al., 1970) for the forward reaction. A value of 15 min^{-1} was calculated for the backward reaction from the Haldane relationship shown in Equation 1.:

$$K_{eq} = \frac{V_f . K_m^q . K_m^p}{V_b . K_m^a . K_m^b} \qquad .....(1)$$

The main feature of a Rapid Equilibrium Random reaction is that the central catalytic step is rate-limiting and the rate of conversion of the central complexes is described by the ratio of $V_f : V_b$ (where V_f and V_b are the maximum velocities for the forward and backward reactions respectively), the individual rate constants veing V_f/E_t (the Turnover Number) for the forward rate constant, and V_b/E_t for the backward reaction. Since the time scale for the simulation was to be in seconds the Turnover Numbers were converted to units of sec^{-1} and these were used as the values of k_f and k_b, i.e. 0.2 sec^{-1} for k_b and 283 sec^{-1} for k_f. As this is the rate limiting step,

Fig. II.4. Citrate Synthase: Rapid Equilibrium Random Bi Bi Reaction Mechanism (Srere, 1972).



Where OAA is oxalacetate, cit is citrate and acCoA is acetylCoA.

the rate constants for the rest of the mechanism are less important. The ratios of $k_{-1}:k_1$, $k_{-2}:k_2$, $k_{-3}:k_3$, $k_{-4}:k_4$, $k_{-5}:k_5$, $k_{-6}:k_6$, $k_{-7}:k_7$ and $k_{-8}:k_8$ correspond to the K_m (K_d) values for the relevant substrate or product binding (Srere, 1972). The kinetic parameters used for evaluation of the rate constants for the mechanism are shown in Table II.1. Reactions simulating the inhibition of citrate synthase by succinylCoA (Smith & Williamson, 1971) were included. Since succinylCoA is a competitive inhibitor of acetylCoA (K_i=0.13mM) two equations were included representing the binding of succinylCoA to the free enzyme and to the enzyme-oxalacetate complex. The rate constants for these equations were such that at a succinylCoA concentration of 0.13mM, the system was at equilibrium.

The entire equation deck as it is used for the simulation is shown in Fig. II.5.

Aconitase (EC. 4.2.1.3)

This enzyme catalyses the conversion of citrate to isocitrate via cis-aconitate thus:

$$\text{citrate} \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} \text{cis-aconitate} \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \text{isocitrate}$$

It has been found in all cells examined for it, and in higher organisms has both mitochondrial and cytoplasmic species. These are thought to be similar as far as mechanism and kinetics are concerned (Eanes & Kun, 1971; Guarrio-Bobyleva *et al.*, 1973). Ferrous ion is necessary for catalytic activity and no other divalent ion can replace this. There is also a ferric ion within the enzyme structure but the function of this is thought to be structural rather than catalytic (Glusker, 1971).

Fig. II.5. Mechanism and Rate Constants used for the Simulation of Citrate Synthase.

$$\text{Enz} + \text{OAA} \underset{5.0\ \text{sec}^{-1}}{\overset{1.0 \times 10^{3}\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.OAA}$$

$$\text{Enz} + \text{AcCoA} \underset{4.5\ \text{sec}^{-1}}{\overset{1.0 \times 10^{3}\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.AcCoA}$$

$$\text{Enz.OAA} + \text{AcCoA} \underset{4.5\ \text{sec}^{-1}}{\overset{1.0 \times 10^{3}\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.OAA.AcCoA}$$

$$\text{Enz.AcCoA} + \text{OAA} \underset{5.0\ \text{sec}^{-1}}{\overset{1.0 \times 10^{3}\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.OAA.AcCoA}$$

$$\text{Enz.OAA.AcCoA} \underset{0.2\ \text{sec}^{-1}}{\overset{283.0\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.Cit.CoA}$$

$$\text{Enz.Cit.CoA} \underset{1.0 \times 10^{3}\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{300.0\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.CoA} + \text{Cit}$$

$$\text{Enz.Cit.CoA} \underset{1.0 \times 10^{3}\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{30.0\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.Cit} + \text{CoA}$$

$$\text{Enz.CoA} \underset{1.0 \times 10^{3}\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{30.0\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz} + \text{CoA}$$

$$\text{Enz.Cit} \underset{1.0 \times 10^{3}\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{300.0\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz} + \text{Cit}$$

$$\text{Enz} + \text{SuCoA} \underset{130.0\ \text{sec}^{-1}}{\overset{1.0 \times 10^{3}\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.I}$$

$$\text{Enz.OAA} + \text{SuCoA} \underset{130.0\ \text{sec}^{-1}}{\overset{1.0 \times 10^{3}\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{Enz*I}$$

Abbreviations: OAA = oxalacetate; AcCoA = acetylCoA; Cit = citrate; SuCoA = succinylCoA.

TABLE II.1. Kinetic Parameters used for Calculation of Rate Constants for the Rapid Equilibrium Random Bi Bi Reaction Mechanism of Citrate Synthase.

Kinetic Constant	Value
$K_m^{oxalacetate}$	5μM
$K_m^{acetylCoA}$	5μM
$K_m^{citrate}$	300μM
K_m^{CoA}	30μM
$V_{max}^{forward}$	17,000 min^{-1}
K_{eq}	4.65×10^5

K_m values are taken from Srere (1972); V_{max} from Singh *et al.* (1970); K_{eq} from Stern *et al.* (1952).

The mechanism of action has been agreed by both kinetic (Henson & Cleland, 1967; Thomson et al., 1966) and mechanistic (Glusker, 1968) studies to be that shown in Fig. II.6. The rate equation for this mechanism was calculated and the kinetic parameters expressed in rate constant form. The known parameters are shown in Table II.2. There were used to solve values for the rate constants using the SIMPLEX program. Fig. II.7 shows the equation deck for aconitase as used for simulations.

Isocitrate Dehydrogenase (EC. 1.1.1.41)

The oxidative decarboxylation of isocitrate is catalysed by the enzyme isocitrate dehydrogenase. The reaction is:

$$\text{isocitrate} + \text{NAD(P)}^+ \rightleftharpoons \text{2-oxoglutarate} + CO_2 + \text{NAD(P)H} + H^+$$

There are two species of the enzyme, one utilising NAD^+, the other $NADP^+$ as cofactor. As was discussed in Chapter I it is most probable that the enzyme concerned with the decarboxylation of isocitrate in the Citric Acid Cycle is the NAD^+ linked enzyme, and this enzyme has been used for the Cycle simulation.

The NAD^+-linked isocitrate dehydrogenase from different sources has different properties. The yeast enzyme is activated by 5'AMP and citrate, and shows positive homotropic effects with isocitrate. The liver enzyme is activated by ADP, AMP and cyclic AMP, while the heart enzyme is activated by ADP only (Plaut, 1970).

For purposes of simulation the data on the heart enzyme were used. The effect of activation by ADP is to lower the apparent K_m for isocitrate and divalent metal ions, to shift the optimum pH to a higher value (Chen & Plaut, 1963), and to aggregate the enzyme protein (Chen et al., 1964). The stimulation is maximal at about 1.0mM ADP (Plaut, 1970). Inhibitors (negative effectors) of the enzyme are NADH and ATP, both being competitive with NAD^+ (Chen & Plaut, 1963).

Fig. II.6. Reaction Mechanism for Aconitase (Glusker, 1968).

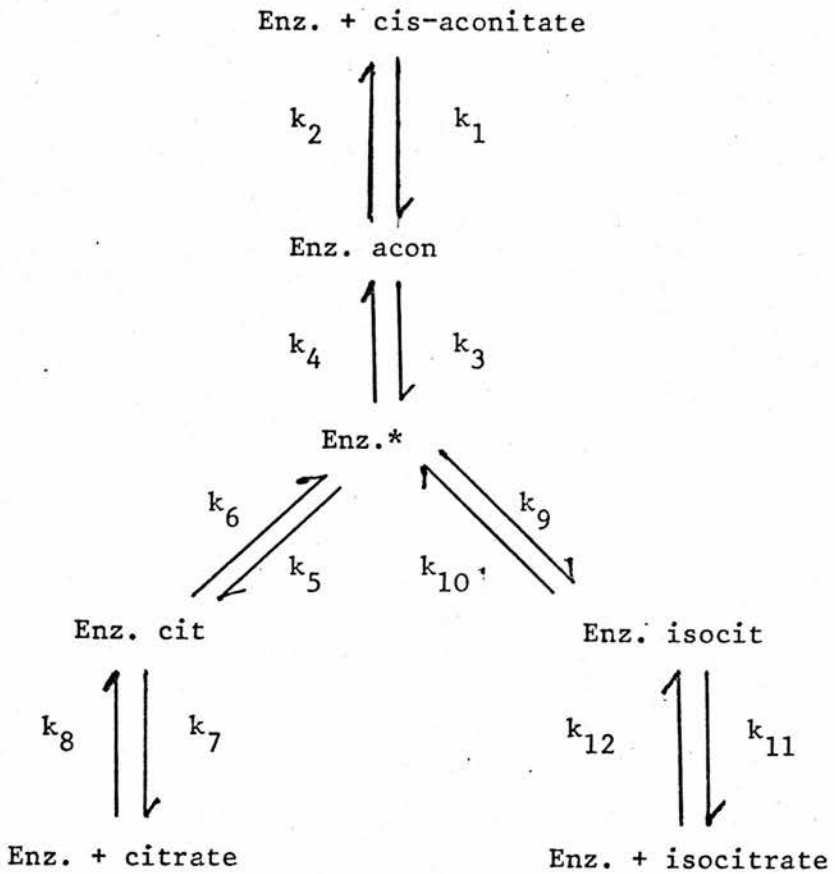


Fig. II.7. Mechanism and Rate Constants used in the Simulation of Aconitase.

$$\text{ENZ} + \text{CIT} \underset{1330.0\ \text{sec}^{-1}}{\overset{550.0\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{ENZ.CIT}$$

$$\text{ENZ.CIT} \underset{65.0\ \text{sec}^{-1}}{\overset{95.0\ \text{sec}^{-1}}{\rightleftharpoons}} \text{ENZ.X}$$

$$\text{ENZ.X} \underset{9200.0\ \text{sec}^{-1}}{\overset{1210.0\ \text{sec}^{-1}}{\rightleftharpoons}} \text{ENZ.ACON}$$

$$\text{ENZ.ACON} \underset{730.0\ \text{mM}^{-1}\ \text{sec}^{-1}}{\overset{360.0\ \text{sec}^{-1}}{\rightleftharpoons}} \text{ENZ} + \text{ACON}$$

$$\text{ENZ.X} \underset{2300.0\ \text{sec}^{-1}}{\overset{2900.0\ \text{sec}^{-1}}{\rightleftharpoons}} \text{ENZ.IC}$$

$$\text{ENZ.IC} \underset{510.0\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{43.0\ \text{sec}^{-1}}{\rightleftharpoons}} \text{ENZ} + \text{IC}$$

Where CIT = citrate; ACON = cis-aconitate; IC = isocitrate.

TABLE II.2. Kinetic Parameters used for Calculation of Rate Constants for Aconitase.

Kinetic Constant	Value
V_{max} (isocit → cit)	21.2 mmol.sec^{-1}.mmol Enzyme^{-1}
V_{max} (acon → cit)	25.6 "
V_{max} (cit → acon)	12.05 "
V_{max} (isocit → acon)	19.7 "
V_{max} (cit → isocit)	13.5 "
V_{max} (acon → isocit)	22.7 "
K_{eq}	3.64×10^{-3}
$K_m^{citrate}$	0.95 mM
$K_m^{aconitate}$	0.099 mM
$K_m^{isocitrate}$	0.139 mM

K_m values and K_{eq} were taken from Thomson et al. (1966). V_{max} values were calculated using the value for V_{max} citrate → isocitrate of 13.5 mmol.sec^{-1}.mmol Enz^{-1} (Villafranca & Mildvan, 1971) and substituting this into the relative V_{max} values measured by Henson & Cleland (1967).

It has been reported (Plaut & Aogaichi, 1968) that the backward reaction i.e. the production of isocitrate from 2-oxoglutarate, is impossible to demonstrate in practice with this enzyme as catalyst. Plaut (1970) has suggested that this is due to a very low binding capacity for CO_2 and 2-oxoglutarate.

The mechanism assumed was Bi-Ter ordered sequential, with NAD^+ binding first, CO_2 being the first product, and NADH the last product to leave the enzyme. The kinetic parameters used for the SIMPLEX procedure are shown in Table II.3. The enzyme was simulated as being in the activated form. It is difficult to interpret how the enzyme might be activated <u>in vivo</u> by ADP since the concentration of the enzyme in mitochondria is much higher than those used for <u>in vitro</u> studies, also it is very likely that enzymes within the mitochondrial matrix are not in true solution (Atkinson, 1969a). These factors make it impossible to predict the distribution of the enzyme in activated and non-activated forms and the rate of conversion between them. Since Cycle simulations were to be carried out under State 3 conditions (i.e. high ADP concentrations), it was assumed that the enzyme is in the activated form. Rate constants for the mechanism were calculated by the SIMPLEX procedure using the kinetic parameters corresponding to the activated enzyme. Also for the optimization process the K_i values for 2-oxoglutarate and CO_2 were set artificially high to take account of the quasi-irreversible nature of the reaction, which otherwise prevents the calculation of a value for K_{eq}.

A reaction to simulate inhibition by ATP was written in by allowing it to bind to the free enzyme, viz:

$$\text{Enz.} + \text{ATP} \underset{150.}{\overset{1000.}{\rightleftharpoons}} \text{Enz. I}$$

TABLE II.3. Kinetic Parameters used for Determination of Rate Constants for NAD^+ linked Isocitrate Dehydrogenase.

Kinetic Parameter	Value
$K_m^{NAD^+}$	0.08 mM
$K_m^{isocitrate}$	0.14 mM
K_i^{NADH}	0.04 mM
K_i^{ATP}	0.15 mM
K_{eq}	855.0
V_{max}	133 $mmol.sec^{-1}.mmol\ Enz^{-1}$
$K_i^{CO_2}$	1.0 M
$K_i^{2\text{-oxoglutarate}}$	1.0 M

All values taken from Plaut (1970) except for $K_i^{CO_2}$ and $K_i^{2\text{-oxoglutarate}}$ which were artificial values used to account for the irreversible nature of the reaction (see text for fuller description).

The simulation was run to check for errors. A slight backward reaction could be demonstrated in the simulations when Plaut & Aogaichi's (1968) original experimental conditions were employed, but the measurement of this in practice would probably be impossible, since the rate of isocitrate production was of the order of 10^{-13} mol/min.

The completed equation deck containing the optimized values of the rate constants for this enzyme is shown in Fig. II.8.

2-Oxoglutarate Dehydrogenase System (EC. 1.2.4.2)

A full description and discussion of this enzyme complex will be given in Chapter IV.

Two simulations of the enzyme were carried out, the first using the information available from the literature up till 1974, and the second using the information gained from the experimental studies reported in Chapter IV. Only the details of the construction of these simulation models will be given here.

For the simulation model based on the published literature on the enzyme, the mechanism used was that of Sanadi (1963) (Fig. II.9). The rate equation describing this mechanism was derived by the method of King & Altman (see Plowman, 1972). The values of $K_m^{(2\text{-oxoglutarate})}$, K_m^{CoA}, $K_m^{NAD^+}$, V_{max} and K_{eq} were taken from Massey (1960) and were used for a SIMPLEX optimization together with the value of $K_m^{(\text{succinylCoA})}$ from Erfle & Sauer (1969). The rate constants calculated by this procedure are shown with the completed equation deck in Fig. II.10.

For the second simulation of this enzyme the mechanism used was that shown as mechanism 5 in Fig. IV.viii.7. The complex constants of the rate equation corresponding to this mechanism (also shown in Fig. IV.viii.7)have been evaluated, and have been used to compute values for the individual rate constants of the mechanism as explained in Chapter IV.

Fig. II.8. Mechanism and Rate Constants used for the Simulation of Isocitrate Dehydrogenase.

$$ENZ + NAD^{+} \underset{4874.0\ sec^{-1}}{\overset{1755.0\ mM^{-1}sec^{-1}}{\rightleftharpoons}} ENZ.NAD^{+}$$

$$ENZ.NAD^{+} + IC \underset{476.0\ sec^{-1}}{\overset{1396.0\ mM^{-1}sec^{-1}}{\rightleftharpoons}} ENZ.X$$

$$ENZ.X \underset{3.8\ mM^{-1}sec^{-1}}{\overset{1181.0\ sec^{-1}}{\rightleftharpoons}} ENZ.Y + CO_2$$

$$ENZ.Y \underset{6.0\ mM^{-1}sec^{-1}}{\overset{1660.0\ sec^{-1}}{\rightleftharpoons}} ENZ.NADH + OG$$

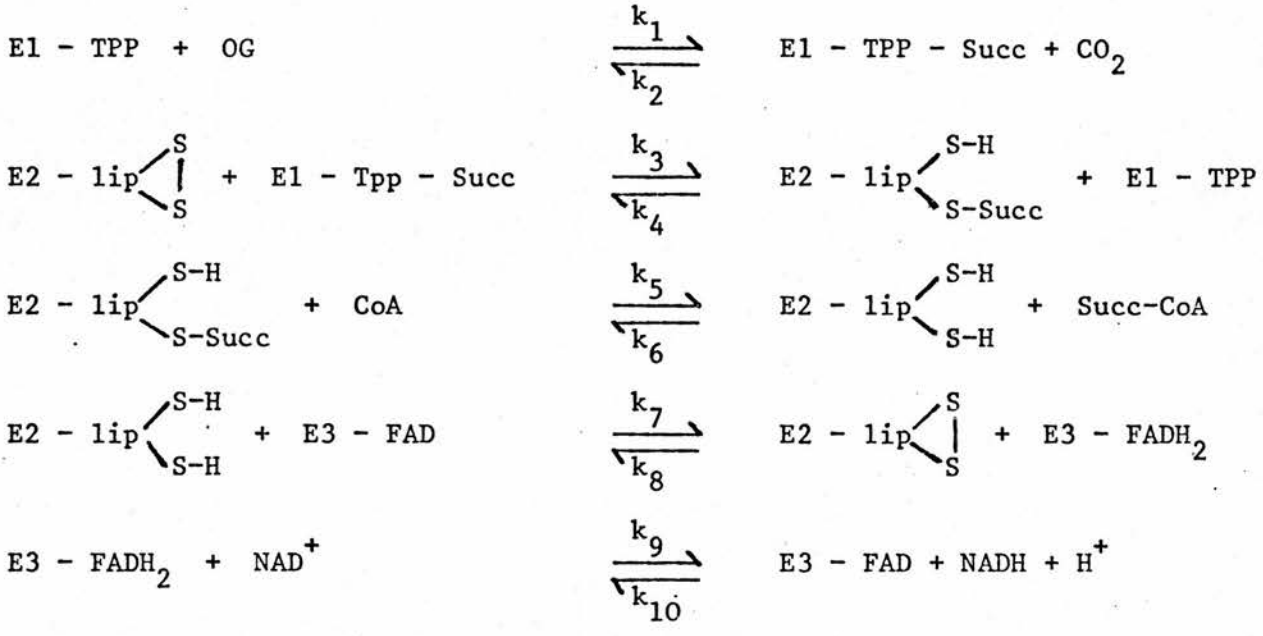
$$ENZ.NADH \underset{4082.0\ mM^{-1}sec^{-1}}{\overset{165.0\ sec^{-1}}{\rightleftharpoons}} ENZ + NADH$$

$$ENZ + ATP \underset{150.0\ sec^{-1}}{\overset{1.0 \times 10^{3}\ mM^{-1}sec^{-1}}{\rightleftharpoons}} ENZI$$

inhibited complex

Where: IC = isocitrate; OG = 2-oxoglutarate.

Fig. II.9. Mechanism Proposed by Sanadi (1963) for the 2-Oxoglutarate Dehydrogenase.



Where OG = 2-oxoglutarate; TPP = Thiamine pyrophosphate; Succ = succinyl residue; $lipS_2$ = lipoic acid; SuccCoA = succinyl CoA; E1,E2,E3 = enzyme forms.

Fig. II.10. Mechanism and Rate Constants used for Simulation of 2-Oxoglutarate Dehydrogenase (Sanadi, 1963).

$$E1 + OG \underset{1.24 \times 10^{5}\ mM^{-1}sec^{-1}}{\overset{4.4 \times 10^{4}\ mM^{-1}sec^{-1}}{\rightleftharpoons}} E2 + CO_2$$

$$E2 \underset{66.0\ sec^{-1}}{\overset{1.73 \times 10^{4}\ sec^{-1}}{\rightleftharpoons}} E3$$

$$E3 + CoA \underset{1.12 \times 10^{4}\ mM^{-1}sec^{-1}}{\overset{5.7 \times 10^{6}\ mM^{-1}sec^{-1}}{\rightleftharpoons}} E4 + SuCoA$$

$$E4 \underset{62.0\ sec^{-1}}{\overset{590.0\ sec^{-1}}{\rightleftharpoons}} E5$$

$$E5 + NAD^{+} \underset{8.35 \times 10^{4}\ mM^{-1}sec^{-1}}{\overset{1.4 \times 10^{5}\ mM^{-1}sec^{-1}}{\rightleftharpoons}} E1 + NADH$$

Where OG = 2-oxoglutarate; SuCoA = succinylCoA. E1-E5 are the various enzyme forms.

Fig. II.11. Mechanism and Rate Constants for the Simulation of 2-Oxoglutarate Dehydrogenase (from experimental results).

$$E + OG \underset{1.22 \times 10^{2}\ sec^{-1}}{\overset{3.51 \times 10^{2}\ mM^{-1}sec^{-1}}{\rightleftharpoons}} E.OG$$

$$E.OG \underset{2.6 \times 10^{-1}\ mM^{-1}\ sec^{-1}}{\overset{2.002 \times 10^{3}\ sec^{-1}}{\rightleftharpoons}} E.X + CO_2$$

$$E.X + CoA \underset{1.88 \times 10^{4}\ sec^{-1}}{\overset{6.58 \times 10^{2}\ mM^{-1}sec^{-1}}{\rightleftharpoons}} E.X.CoA$$

$$E.X.CoA \underset{2.6\ mM^{-1}sec^{-1}}{\overset{3.12 \times 10^{4}\ sec^{-1}}{\rightleftharpoons}} E.Y + SuCoA$$

$$E.Y + NAD^{+} \underset{2.18 \times 10^{1}\ sec^{-1}}{\overset{8.58 \times 10^{2}\ mM^{-1}sec^{-1}}{\rightleftharpoons}} E.Y.N$$

$$E.X + NAD^{+} \underset{8.06 \times 10^{-1}\ sec^{-1}}{\overset{1.768 \times 10^{3}\ mM^{-1}sec^{-1}}{\rightleftharpoons}} E.X.N$$

$$E.X.N + CoA \underset{3.07 \times 10^{4}\ sec^{-1}}{\overset{3.08 \times 10^{5}\ mM^{-1}sec^{-1}}{\rightleftharpoons}} E.X.N.C$$

$$E.X.N.C \underset{1.534 \times 10^{3}\ mM^{-1}sec^{-1}}{\overset{1.144 \times 10^{3}\ sec^{-1}}{\rightleftharpoons}} E.Y.N + SuCoA$$

$$E.Y.N \underset{2.09 \times 10^{6}\ sec^{-1}}{\overset{1.82 \times 10^{2}\ sec^{-1}}{\rightleftharpoons}} E.NADH$$

$$E.NADH \underset{4.46 \times 10^{4}\ mM^{-1}sec^{-1}}{\overset{1.06 \times 10^{6}\ sec^{-1}}{\rightleftharpoons}} E + NADH$$

Where: OG = 2-oxoglutarate; SuCoA = succinylCoA.

The complete equation deck for this version of the enzyme mechanism is shown in Fig. II.11.

Succinyl Thiokinase (EC. 6.2.1.4)

This enzyme catalyses the reaction:

$$\text{succinylCoA} + \text{GDP} + \text{Pi} \rightleftharpoons \text{succinate} + \text{CoA} + \text{GTP}$$

The enzyme from plant, bacterial and some insect sources is specific for ADP (ATP) (Kaufman & Alivistos, 1955; Smith et al., 1957; Hansford, 1973).

Investigations on the mechanism of the enzyme have been hampered by the difficulties of purifying large quantities of it, and by the complexity of the reaction catalysed. The mechanism shown in Fig. II.12a was first proposed for the enzyme (Hager, 1962). Later Benson, Robinson and Boyer (1969) showed by isotope exchange studies that a coacylated form of the enzyme did not participate in the major pathway of catalysis. More recent investigation suggests that succinyl phosphate is an intermediate (Hildebrand & Spector, 1969). This mechanism is shown in Fig. II.12b. Common to both mechanisms is the existence of a phosphorylated enzyme form which binds GDP. GTP is subsequently formed and released. It has been established that the enzyme is phosphorylated at a particular histidine residue (Mitchell et al., 1964; Bridger et al., 1968; Grinnell & Nishimura, 1969b). Investigations of the structure of both the E. coli (Bridger, 1971) and pig heart (Brownie & Bridger, 1972) enzymes show that the enzyme consists of two types of subunit, two of each in the E. coli enzyme and one of each in the pig heart species. The subunits have molecular weights of approximately 42,500 and 34,500 daltons, respectively. The histidine residue which is phosphorylated during catalysis is contained within the smaller subunit in both enzymes (Brownie & Bridger, 1972).

Fig. II.12. Proposed Reaction Mechanisms for Succinyl Thiokinase.

a) Mechanism Proposed by Hager (1962)

$$\text{Enz} + \text{succinyl CoA} \rightleftharpoons \text{Enz} \sim \text{CoA} + \text{succinate}$$

$$\text{Enz} \sim \text{CoA} + P_i \rightleftharpoons \text{Enz} \sim P_i + \text{CoA}$$

$$\text{Enz} \sim P_i + \text{GDP} \rightleftharpoons \text{Enz} + \text{GTP}$$

b) Mechanism Proposed by Hildebrand & Spector (1969)

$$\text{Enz} + \text{succinyl CoA} + P_i \rightleftharpoons \text{Enz.succinyl} \sim P_i + \text{CoA}$$

$$\text{Enz.succinyl} \sim P_i \rightleftharpoons \text{Enz} \sim P_i + \text{succinate}$$

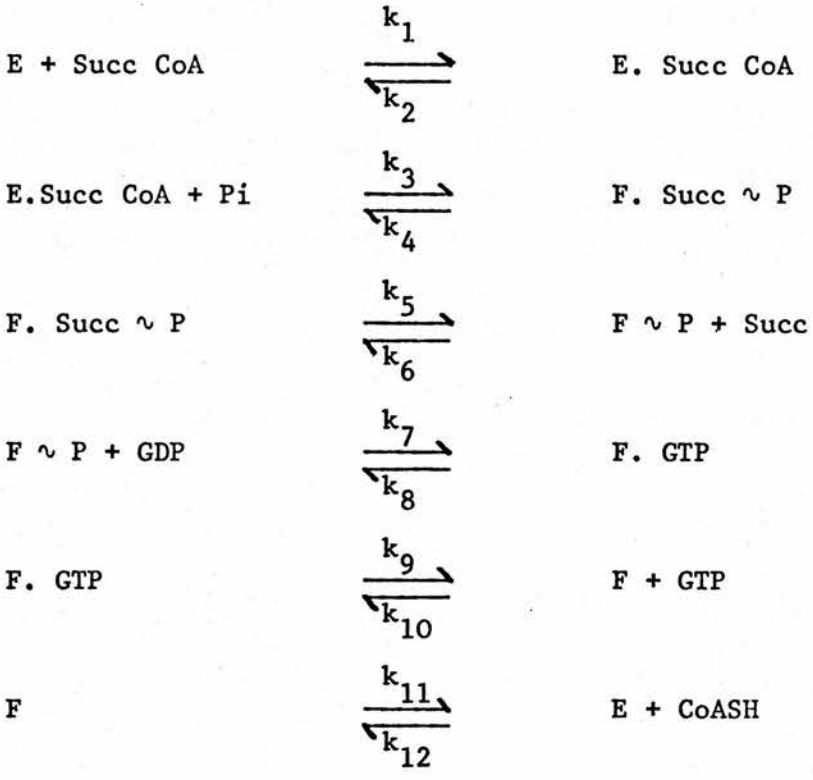
$$\text{Enz} \sim P_i + \text{GDP} \rightleftharpoons \text{Enz} + \text{GTP}$$

Where P_i = phosphate.

Kinetic studies on these enzymes have been carried out by various workers (Cha & Parks, 1964; Moffet & Bridger, 1970). The results have not generally been in agreement with the findings of the mechanistic and structural work on the enzyme. This may in part be due to the fact that it catalyses a complex reaction. It has also been suggested that CoA is an effector of the enzyme. It has been demonstrated (Moyer et al., 1967; Grinnell & Nishimura, 1969[a]) that CoA, although not forming a high energy intermediate form with the enzyme as was proposed in the original mechanism (Fig. II.12a), does nevertheless bind to it, and the presence of CoA at this 'allosteric site' appears to have an accelerating influence on phosphorylation of the enzyme.

The state of knowledge about this catalyst is thus not very conclusive and no definite kinetic or catalytic mechanism has been proposed. In order to carry out a simulation of this enzyme, the mechanism shown in Fig. II.13 was adopted. Although this is not a generally accepted mechanism, it contains many of the essential reactions known to occur in the presence of the enzyme. A reaction in which CoA binds to the free enzyme, converting it to a different form (E*), was included since it was considered this may be an important feature of the mechanism, especially with a view to examining control properties of the Citric Acid Cycle. Although the mechanism shown in Fig. II.13 cannot be considered to be the most accurate possible representation of the overall enzymic reaction, it does contain the important elements of the catalytic behaviour of the heart enzyme, and was thought to be the best description for simulation purposes which could be obtained from the information available at present.

Fig. II.13. Reaction Mechanism for Succinyl Thiokinase adopted for Simulation Studies.



See text for details of how this mechanism was constructed.

The rate equation corresponding to this mechanism was calculated by the King and Altman method and is shown in Fig. II.14. The published values of the kinetic parameters were used in the SIMPLEX procedure previously described, to find estimates of the values for the individual rate constants. The completed equation deck is shown in Fig. II.15. As with the previous enzymes the equation deck was set up and the simulation run to check for possible errors.

Succinate Dehydrogenase (EC. 1.3.99.1)

The oxidation of succinate is catalysed by the enzyme Succinate Dehydrogenase thus:

$$\text{succinate} + \text{F.P.}_{ox} \rightleftharpoons \text{fumarate} + \text{F.P.}_{red}$$ (where F.P. = flavoprotein).

Kinetic studies of the enzyme have been carried out by many workers. Reported values for kinetic constants have varied quite a great deal, and this has been accounted for by the activation characteristics and the use of different electron acceptors to assay the reaction (Singer *et al.*, 1973). The actual electron acceptor *in vivo* is not known although it is generally thought to be coenzyme Q (Rossi *et al.*, 1970).

The major problem with the simulation of this enzyme was to decide whether it catalyses a reversible reaction. One enzyme preparation can catalyse both the forward and backward reactions, but a very different electron donor/acceptor has to be used for each. The reaction *in vivo* has been reported to be reversed during porphyrin synthesis in rat liver, but the backward reaction appears to be brought about by an increased $NADH:NAD^+$ ratio (Labbe *et al.*, 1965). This would not represent a truly reversible reaction of Succinate Dehydrogenase since the enzyme does not directly involve the pyridine nucleotides. It is also true that these measurements were made in rats with drug-induced porphyria, and their livers were behaving in an unphysiological fashion. It is still a debatable point whether the succinic dehydrogenase of heart tissue under normal conditions can catalyse a reversible reaction.

Fig. II.14. Succinyl Thiokinase: Rate Equation for Mechanism shown in Fig. II.13.

$$V = \frac{(k_1k_3k_5k_7k_9k_{11}A.B.C - k_2k_4k_6k_8k_{10}k_{12}P.Q.R)Et}{k_2k_7k_9k_{11}(k_4+k_5)C + k_2k_4k_6k_{11}(k_8+k_9)P + k_1k_3k_5k_{11}(k_8+k_9)A.B.}$$

$$+ k_1k_7k_9k_{11}(k_4+k_5)A.C. + k_3k_5k_7k_9k_{11}B.C. + k_2k_4k_6k_8k_{10}P.Q.$$

$$+ k_2k_4k_6k_{12}(k_8+k_9)P.Q. + k_2k_8k_{10}k_{12}(k_4+k_5)Q.R. + k_1k_3k_7(k_9k_{11}+k_5k_{11}+k_5k_9)A.B.C.$$

$$+ k_6k_{10}k_{12}(k_4k_8+k_3k_8+k_2k_4)P.Q.R + k_1k_4k_6k_{11}(k_8+k_9)A.P. + k_1k_4k_6k_8k_{12}B.Q.R$$

$$+ k_2k_7k_9k_{12}(k_4+k_5)C.R + k_1k_3k_5k_8k_{10}A.B.Q + k_2k_7k_{10}k_{12}(k_4+k_5)C.Q.R$$

$$+ k_3k_5k_7k_9k_{12}B.C.R + k_3k_6k_8k_{10}k_{12}B.P.Q.R. + k_1k_3k_6k_8k_{10}A.B.P.Q$$

$$+ k_3k_5k_7k_{10}k_{12}B.C.Q.R$$

Where: A = succinyl CoA, B = Pi, C = GDP, P = succinate, Q = GTP, R = CoA. Equation calculated using the method of King & Altman.

Fig. II.15. Mechanism and Rate Constants used for Simulation of Succinyl Thiokinase.

$$\text{Enz} + \text{succinylCoA} \underset{11.4\ \text{sec}^{-1}}{\overset{370\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.sCoA}$$

$$\text{Enz.sCoA} + \text{Pi} \underset{65.2\ \text{sec}^{-1}}{\overset{43.8\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{Enz} * \text{SuP}$$

$$\text{Enz} * \text{SuP} \underset{24.0\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{66.7\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz} * \text{P} + \text{succinate}$$

$$\text{Enz} * \text{P} + \text{GDP} \underset{27.2\ \text{sec}^{-1}}{\overset{2268\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{Enz} * \text{GTP}$$

$$\text{Enz} * \text{GTP} \underset{40804\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{1217\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz} * + \text{GTP}$$

$$\text{Enz} * \underset{547.4\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{13.4\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz} + \text{CoA}$$

Where Pi = phosphate; SuP = succinyl phosphate; sCoA = succinylCoA.

For purposes of simulation the enzyme has in fact been assumed to catalyse an irreversible reaction. The mechanism and rate constants used are taken from (Zeilemaker *et al.*, 1969a). This is only a 'minimal' mechanism and a more complex sequence of events cannot be ruled out. However it contains all the essential details of the catalysis and is acceptable for simulation purposes, as SDH is not thought to be an important controlling enzyme, even by Singer (loc. cit.).

Since the actual electron acceptor involved with the enzyme *in vivo* has not been established, a hypothetical electron acceptor (A) has been used in the simulation. The concentration of 'A' and the ratio of A_{ox}:A_{red} used will be discussed later.

Although OAA is a potent inhibitor of succinic dehydrogenase *in vitro* (Zeilemaker *et al.*, 1969b) it was decided not to include this in the simulation. This is more fully discussed in Chapter I.

The simulation of the enzyme worked satisfactorily in isolation.

Fumarase (EC. 4.2.1.2)

This enzyme was first purified from pig heart muscle by Massey in 1952. The reaction catalysed is the simplest in the cycle:

fumarate + H_2O $\rightleftharpoons$ L - malate

The kinetics and mechanism of the reaction are very well established, the only complication being the existence of two ionisable groups on the protein rendering the reaction rather sensitive to pH.

The enzyme is inhibited by a number of dicarboxylic and some tricarboxylic acids. The inhibition is competitive, and has been used as evidence that attachment of the substrate to the enzyme is through the two carboxylic acid groups (Massey, 1953). Another competitive inhibitor is ATP (Penner & Cohen, 1969), however the physiological significance of this is questionable since the magnesium chelate of ATP is not inhibitory.

The enzyme also exhibits negative cooperativity at high substrate concentrations. This has been proposed to be a Flip - Flop control mechanism (Lazdunski et al., 1971), and since the enzyme consists of four identical subunits (Hill & Kanarek, 1964) this is a distinct possibility.

For the simulation the mechanism adopted was that proposed by Alberty & Pierce (1957) and the minimum values of the rate constants for the mechanism reported by these workers were used. These are shown in Fig. II.16. The simulation was carried out and found to be satisfactory.

Fig. II.16. Mechanism and Rate Constants used for the Simulation of Fumarase.

$$\text{Enz} + \text{fumarate} \underset{2.7 \times 10^{4}\ \text{sec}^{-1}}{\overset{1.1 \times 10^{7}\ \text{sec}^{-1}\text{mM}^{-1}}{\rightleftharpoons}} \text{Enz.fumarate}$$

$$\text{Enz.fumarate} + H_2O \underset{1.7 \times 10^{3}\ \text{sec}^{-1}}{\overset{2.3 \times 10^{3}\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.malate}$$

$$\text{Enz.malate} \underset{5 \times 10^{6}\ \text{sec}^{-1}\text{mM}^{-1}}{\overset{4.6 \times 10^{4}\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz} + \text{malate}$$

Malate Dehydrogenase (EC. 1.1.1.37)

This enzyme catalyses the final stage of the cycle i.e. the oxidation of L-malate to oxalacetate with the concomitant formation of NADH + H^+ from NAD^+. The equilibrium of the reaction lies very much to the side of malate and NAD^+, the equilibrium constant being in the range of 10^{-4} (Raval & Wolfe, 1962a).

There are two species of the enzyme, mitochondrial and cytoplasmic. As far as possible, data for use in the simulation of the cycle was for the mitochondrial species, since the two species do differ markedly in their kinetic properties and molecular weight (Lowenstein, 1967).

The enzyme has been shown (Raval & Wolfe, 1962a) to have a compulsory binding order of substrates, with NAD^+ (NADH) binding first. Later work suggested that abortive complexes are formed (Silverstein & Sulebele, 1969a,b) and that there may be isomerisation of the enzyme - NAD^+ complex (Heyde & Ainsworth, 1968). An ordered sequential mechanism has never been in dispute. Rate constants for the mechanism were calculated by Raval & Wolfe (1962b) and are shown in Fig. II.17. For simulation purposes this mechanism and rate constants were used. There has been some criticism of these values and of this mechanism by Heyde & Ainsworth (1968), who showed that with their measured kinetic values, one calculated rate constant has a negative value. They suggested that this is due to an inadequacy in the mechanism. However, since the results of Raval & Wolfe (1962b) are the most complete set of data available and since the rate constants calculated by Raval & Wolfe correspond to the values of the kinetic constants quite accurately, it was considered that they were the best available choice for simulation purposes.

Fig. II.17. Mechanism and Rate Constants used for the Simulation of Malate Dehydrogenase.

$$\text{Enz} + \text{NAD}^+ \underset{5.7 \times 10^2\ \text{sec}^{-1}}{\overset{5.5 \times 10^2\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.NAD}^+$$

$$\text{Enz.NAD}^+ + \text{malate} \underset{3.3 \times 10^4\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{2.0 \times 10^3\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \begin{matrix}(\text{Enz.NAD}^+\text{.mal}) \\ (\text{Enz.NADH.OAA})\end{matrix}$$

$$\text{Enz.NADH.OAA} \underset{1.7 \times 10^4\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{8.3 \times 10^3\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.NADH} + \text{OAA}$$

$$\text{Enz.NADH} \underset{3.3 \times 10^4\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{1.7 \times 10^2\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz} + \text{NADH}$$

Where OAA = oxalacetate; mal = malate.

There are no known metabolic inhibitors of the enzyme of any physiological significance. It has been suggested that a Flip - Flop or Half Sites Reactivity control is operative with this enzyme (Harada & Wolfe, 1968; Lazdunski, 1972). This does not affect the Michaelian kinetics and has been challenged by Silverstein & Sulebele (1969b). For these reasons it was decided not to make any allowances for this in the simulation.

iv) Simulation of Additional Enzymes

3-Keto acid CoA Transferase (EC 2.8.3.5)

The activity of this enzyme was first noted in 1953 by Green et al. and by Stern et al. (1953). The enzyme catalyses the exchange of a CoA residue between the two acyl groups of succinate and acetoacetate, thus

Succinyl CoA + Acetoacetate $\rightleftharpoons$ acetoacetylCoA + succinate

The mechanism of the reaction has been established by kinetic, isotope exchange and isotope tracer studies (Hersh & Jencks, 1967a; Blair, 1969; Solomon & Jencks, 1969; Benson & Boyer, 1969) to be Ping Pong Bi Bi as shown in Fig. II.18. The coacylated enzyme form has been isolated and characterized (Hersh & Jencks, 1967b).

This enzyme is found in all tissues except the liver. It is a necessary enzyme for the utilisation of ketone bodies by the cell and apparently it is most active in the heart of most species (Tildon & Sevdalian, 1972). This is not surprising since it has been well established that heart tissue preferentially oxidises ketone bodies (Barnes et al., 1938; Hall, 1961; Williamson & Krebs, 1961; Olsen, 1962; Little et al., 1970).

For purposes of obtaining rate constants for the mechanism in Fig. II.18, the kinetic values reported by Hersh & Jencks (1967a) were used. These were used for a SIMPLEX optimisation and the rate constants

Fig. II.18. Mechanism and Rate Constants for Simulation of 3-Keto Acid CoA Transferase.

$$\text{Enz} + \text{succinylCoA} \underset{3108\ \text{sec}^{-1}}{\overset{669\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.succinylCoA}$$

$$\text{Enz.succinylCoA} \underset{26.3\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{41.85\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz} \sim \text{CoA} + \text{succinate}$$

$$\text{Enz} \sim \text{CoA} + \text{acac} \underset{1334\ \text{sec}^{-1}}{\overset{906.4\ \text{mM}^{-1}\text{sec}^{-1}}{\rightleftharpoons}} \text{Enz.acacCoA}$$

$$\text{Enz.acacCoA} \underset{1632.6\ \text{mM}^{-1}\text{sec}^{-1}}{\overset{346\ \text{sec}^{-1}}{\rightleftharpoons}} \text{Enz} + \text{acacCoA}$$

Where acac = acetoacetate and acacCoA = acetoacetyl CoA.

obtained are also shown in Fig. II.18. They give a very good correlation between experimental and theoretical values of the kinetic parameters.

Acetyl CoA Acetyl Transferase (CoA Thiolase) (EC 2.3.1.9)

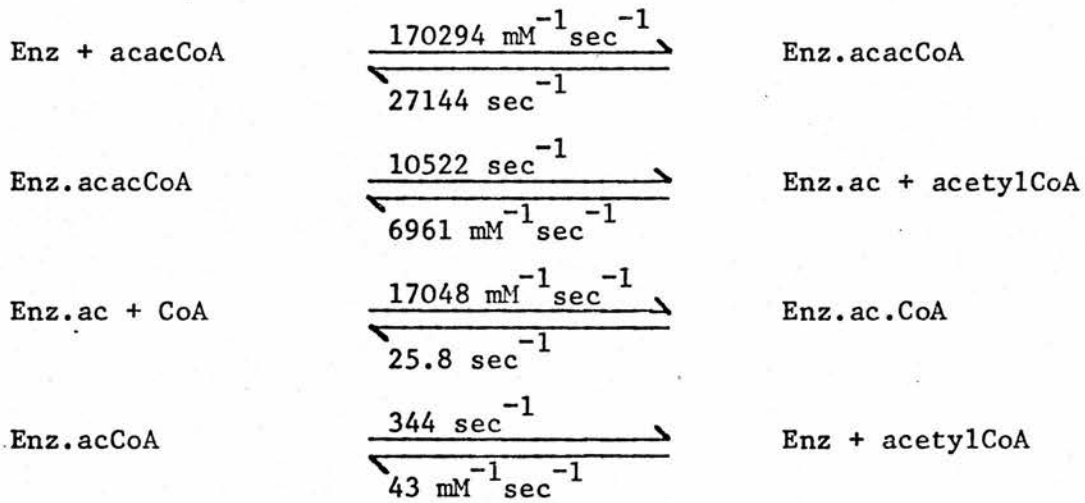
The activity of this enzyme was noted during the study of fat metabolism and ketone body utilisation (Lynen, et al., 1952; Lynen & Ochoa, 1953). The reaction catalysed is the formation of two AcetylCoA units from acetoacetylCoA and CoA.

There are two enzymes which catalyse this reaction, one which is specific for acetoacetylCoA only, and the other which can also cleave C_6 units to acetylCoA and acetoacetylCoA. The latter enzyme is part of the system of fatty acid oxidation, while the former is the enzyme involved in ketone body utilisation, and is therefore the enzyme considered for simulation purposes.

Structural studies on the heart enzyme indicate that it is composed of four identical polypeptide chains, each with one active site (Gehring & Harris, 1970). The molecular weight of the entire complex is 170,000.

Until recently (Middleton, 1971; Kornblatt & Rudney, 1971) it had not been realised that there were two species of the enzyme, one cytoplasmic and one mitochondrial. It has been suggested from kinetic measurements that the mitochondrial enzyme is that primarily utilised in ketone body oxidation (Middleton, 1973). Thus for a simulation study of the Citric Acid Cycle and the oxidation of ketone bodies by the Cycle, the mitochondrial species of the enzyme would be the one to simulate. Unfortunately, kinetic data for this species is incomplete, however by taking the known values for the heart mitochondrial enzyme and supplementing them with values from the liver mitochondrial enzyme (Middleton, 1973), enough data for estimating rate constants can be gained. The mechanism of the enzyme was taken

Fig. II.19. Mechanism and Rate Constants used for Simulation of AcetylCoA Acetyl Transferase.



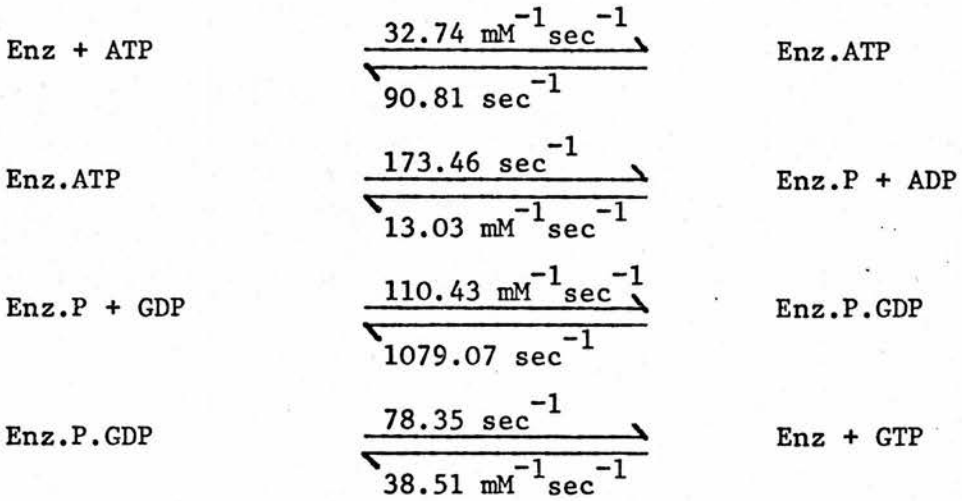
Where acacCoA = acetoacetylCoA.

TABLE II.4. Kinetic Parameters used for Calculation of Rate Constants for Acetyl CoA Acetyl Transferase.

Parameter	Value	Source of Enzyme
V_{max}	333 sec^{-1}	pig heart
$K_m^{acetoacetylCoA}$	0.007 mM	rat liver
K_m^{CoA}	0.021 mM	rat liver
K_{eq}	5×10^4	pig heart

V_{max} was taken from Gehring & Lynen (1972), K_m values from Middleton (1973) and K_{eq} from Stern *et al.* (1953).

Fig. II.20. Mechanism and Rate Constants used for Simulation of Nucleoside Diphosphate Kinase.



Where P is inorganic phosphate.

TABLE II.5. Kinetic Constants used in the Calculation of the Rate Constants for Nucleoside Diphosphate Kinase.

Parameter	Value
K_m^{ATP}	1.4 mM
K_m^{ADP}	0.1 mM
V_{max} (ADP→ATP)	45.8 sec^{-1}
V_{max} (GDP→GTP)	11.4 sec^{-1}
K_{eq}	1.0

K_m and V_{max} values from Colomb *et al.* (1969), K_{eq} from Parks & Agarwal (1973).

as Ping Pong Bi Bi as suggested by Gehring & Lynen (1972). Taking the kinetic parameter values shown in Table II.4, rate constants were calculated for this mechanism. The lack of data did not allow the rate constants to be ascertained within very strict limits, and a range of sets of rate constants can be found which fit the data. One of these sets was chosen by the criteria previously given. The entire equation deck as used in the simulation is shown in Fig. II.19.

Nucleoside Diphosphate Kinase (EC. 2.7.4.6)

The activity of this enzyme is demonstrable in most tissues, although there are different patterns on electrofocussing the enzyme from different subcellular fractions and from different tissues (Cheng et al., 1973). An enzyme catalysing the exchange of phosphate between guanine and adenine nucleotides:

$$GTP + ADP \rightleftharpoons GDP + ATP$$

has been isolated from beef heart mitochondria (Colomb et al., 1969), and displays a Ping Pong Bi Bi reaction mechanism.

For simulation purposes the K_{eq} was taken to be 1.0 (Parks & Agarwal, 1973) and the kinetic data were taken from Colomb et al. (1969) and are shown in Table II.5. These were used to calculate rate constants using the SIMPLEX procedure. The completed equation deck is shown in Fig. II.20.

v) The Complete Citric Acid Cycle Model

With the simulation of the component enzymes of the Cycle complete, a number of additions have to be made before a complete model of the Cycle can be constructed.

Firstly, intramitochondrial concentrations of the enzymes, coenzymes and intermediates have to be established. The concentrations of the enzymes were either taken from Srere (1968) or calculated by the method described therein, except for succinyl thiokinase and 3-keto acid CoA transferase whose concentrations could not be calculated because the activities in the tissue are unknown. These enzymes were given an assumed concentration of 0.01mmoles per kg mitochondria. The enzyme concentrations used are shown in Table II.6. The total concentrations of the coenzymes were taken from Williamson & Corkey (1969) and are expressed as mmoles per kg mitochondria. The ratios of the various forms of the coenzymes were however varied according to the investigations being performed. Substrate concentrations were also adapted from Williamson & Corkey (1969), although these were used merely as starting values and were allowed to vary according to the simulation, to new steady state values. The difference between input and steady state concentrations of the substrates was in some cases quite large but since the values quoted by Williamson & Corkey are for cellular contents and the Cycle is a mitochondrial pathway, the differences did not cause great concern.

All concentrations were expressed as millimoles per kilogram mitochondria, following Srere's (1968) method for calculating enzyme concentrations. Williamson & Corkey's data can easily be converted to the same units. In all calculations the assumption of Srere (1968) that the mitochondria represent 20% of the cellular contents has been used, although in the light of the work of Page & McCallister (1973) it would appear that for heart tissue a figure of 36% would be more accurate. Rather than recalculate all the concentrations and rerun the simulation, the original values have been used. However since all concentrations were calculated using the same assumptions and

TABLE II.6. Enzyme Concentrations used in the Simulation of the Citric Acid Cycle.

Enzyme	Concentration (mmol/Kg mitochondria)	Source of value
Citrate Synthase	0.026	Srere (1968)
Aconitase	0.074	see Chapter III
Isocitrate Dehydrogenase	0.092	Srere (1968)
2-Oxoglutarate Dehydrogenase	0.006	Srere (1968)
Succinyl Thiokinase	0.01	Estimated
Succinic Dehydrogenase	0.0023	see Chapter III
Fumarase	0.008	Srere (1968)
Malate Dehydrogenase	0.07	Srere (1968)
3-Keto Acid CoA Transferase	0.01	Estimated
AcetylCoA Acetyl Transferase	0.00465	see Chapter III
Nucleoside Diphosphate Kinase	0.0044	see Chapter III

thus are in error by the same amount this was not considered to have any effect on the conclusions drawn from the results. The measured control parameters (Sensitivities and Elasticities) should in no way be affected by this. The steady state flux rates quoted will be rather lower than they should be per kg of heart mitochondria.

The completion of the Cycle model also requires the addition of reactions simulating the input of acetylCoA, the regeneration of free CoA, the removal of CO_2, the oxidation of NADH and the reduced acceptor from succinic dehydrogenase, to control the ratios of ATP:ADP and GTP:GDP and to stabilise the concentration of phosphate. The reactions used to fulfil these functions are shown in Fig. II.21.

Inclusion of such 'dummy' reactions is necessary before simulation, since without the specification of these parameters,the Citric Acid Cycle could not operate. These reactions represent much more complex metabolic processes on which the Citric Acid Cycle in vivo is dependent, but simulation of these processes is unnecessary at this stage, since the investigation is aimed at elucidating control mechanisms which operate at the level of the Cycle itself.

The rate constants for such reactions are such that they do not become rate limiting steps for the simulation, i.e. these reactions have sufficient capacity to respond to any increase in the supply or demand caused by changes made within the Cycle. For this purpose the rate constants and concentrations of the 'dummy' enzymes (PREC and REM, see Fig. II.21) were given relatively high values, so that for reversible reactions the net flux is very small compared to either forward or backward fluxes.

Where the simulation model was used to examine the effects on the Cycle of varying the rate of acetylCoA supply and NADH reoxidation, a different type of 'dummy' reaction was used. The reactions

Fig. II.21. 'Dummy' Reactions to Control the Conditions of Simulation of the Citric Acid Cycle.

1.0 PREC	$\underset{33}{\overset{1000}{\rightleftharpoons}}$	1.0 PREC + AcCoA
1.0 REM + CoA	$\underset{1000}{\overset{66}{\rightleftharpoons}}$	1.0 REM
CO_2	$\xrightarrow{100}$	Bicarb
NADH	$\underset{53}{\overset{47}{\rightleftharpoons}}$	NAD^+
Aox	$\underset{50}{\overset{50}{\rightleftharpoons}}$	ARED
ATP	$\underset{10}{\overset{470}{\rightleftharpoons}}$	ADP + Pi
ATP + GDP	$\underset{100}{\overset{100}{\rightleftharpoons}}$	ADP + GTP

All rate constants are in terms of sec^{-1} or mmol.per kg mito. sec^{-1}. PREC and REM are 'artificial' enzymes producing acetylCoA and removing CoA respectively. Aox is the hypothetical electron acceptor for succinic dehydrogenase.

used were irreversible with a rate constant calculated such that the flux through the reaction was the same as the Cycle flux at steady state under 'normal' conditions (normal in the case refers to the standard conditions used for the Cycle simulations). Implications of the use of these equations will be more fully discussed in Chapters IV and V.

CHAPTER III

METHODS

Section I. Mathematical Methods

i) Simplex Optimization Procedure

Optimization means the provision of a set of numerical parameter values which will give the best fit to an equation or series of equations, or to a set of data. If the equations are linear with respect to the variables, the best fit can be achieved by minimizing the sum of the squares, i.e., finding the values of the variables at the minimum point of the sum of squares function. If, for example, this function is represented by the equation:

$$S = \sum_{i=1}^{n} \left[y_{est_i} - (a + bx_i) \right]^2 \qquad \ldots\ldots (1)$$

on differentiating with respect to the parameters a and b, the resulting partial differential equations are:

$$\frac{\partial S}{\partial a} = \sum_{i=1}^{m} (-2y_i + 2a) \qquad \ldots\ldots (2)$$

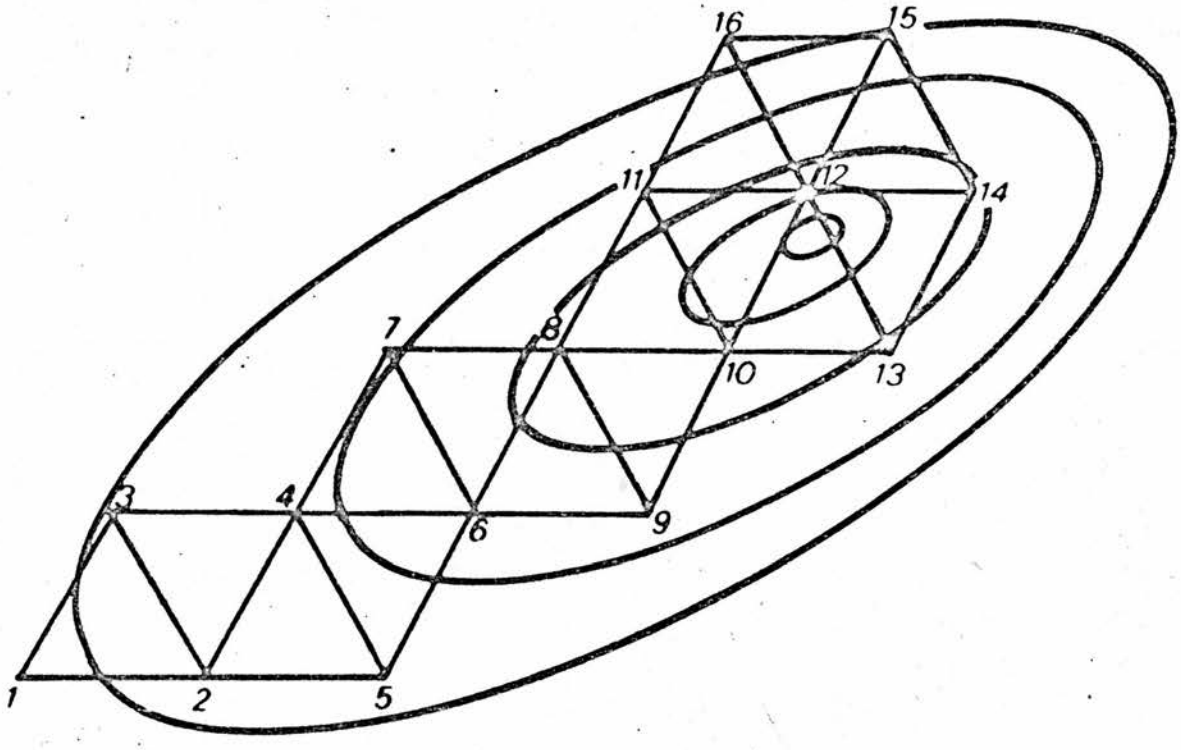
$$\frac{\partial S}{\partial b} = \sum_{i=1}^{n} (-2x_i y_i + 2bx_i^2) \qquad \ldots\ldots (3)$$

When both (2) and (3) are set to zero, these conditions ensure that S is at a minimum turning point for the function, and values for a and b can be found by solving the simultaneous equations (2) and (3). However, where values are to be fitted to more complex equations, this method may be impossible to apply, as differentiation may not be possible, or it can produce non-linear partial differential equations which can be solved only with great difficulty.

Minimizing functions of a single variable by this method is also impossible. Various optimization methods have been introduced to cope with this type of problem (Beveridge & Schechter, 1970). Such methods can also be used to solve the more difficult situations by treating the sum of squares as the single variable to be minimized. The sum of squares is a function of all the equations containing the parameters and the experimental observations, that is, the sum of all the squared differences (or absolute deviations) between the theoretical and experimental values of the equations.

One optimization technique that has been developed for this purpose is 'SIMPLEX'. It was introduced by Spendley, Hext and Hinsworth (1962) and improved by Nelder & Mead (1965). Where there are n parameters to be fitted, an initial estimate for each is supplied together with a 'step length' which is the search area for an improved estimate. The $n+1$ vertices of a simplex figure are calculated from this information, such that if $n=2$ and the parameters to be estimated are say, x and y, the simplex figure has three vertices. Values of the function at these vertices are calculated with x, y; x',y; and x,y'; where x'=x + step length and y' = y + step length. The vertex which has the worst (largest) sum of squares is then reflected through the hyperplane of the remaining vertices forming a new vertex and thus a new figure. The process is then repeated with the new figure; however, if the worst vertex is the newest one the second worst vertex is reflected. This avoids a situation where the optimization oscillates between two simplexes, and allows the procedure to change to a more favourable direction. An illustration of the progress of a simplex procedure is shown in Fig. III.1., for the fitting of two parameters i.e. a triangular simplex. Once near the minimum effort is concentration on locating the best

Fig.III.1. Schematic Representation of a Simplex Procedure for the fitting of two Parameters.



Diagrammatic representation of the progress of a SIMPLEX procedure, for fitting two parameters, towards a minimum situated within an elliptical depression. (The axes x and y for this diagram would be at 60^{o} to each other.)

minimum possible. In the figure illustrated (Fig. III.1), once point 12 has been found, the search will be drawn into a smaller area around this point in an attempt to find a better minimum. This continues until the 'best minimum' is found, i.e. where no set of parameter estimates can be found that give a better fit.

With this method, if the functions are well defined then a GLOBAL minimum can be reached i.e. no other set of parameter estimates can be found which give a lower value for the sum of squares. The 'Simplex' method is also robust in that it can reach a minimum from almost any starting coordinates (Davis & Ottaway, 1972).

There are many other methods designed for the optimization of functions of a single variable, for example Powell's minimization (Powell, 1964) or the 'conjugate gradients' method of Fletcher & Reeves (1964). Surveys of the types of non-linear optimizations have been published (Swann, 1969; Beveridge & Schechter, 1970). Most of these procedures require fairly accurate initial estimates or require that the estimates are accurately scaled to the true values. As yet however, no optimization procedure has been devized which is guaranteed to reach a 'Global Minimum'.

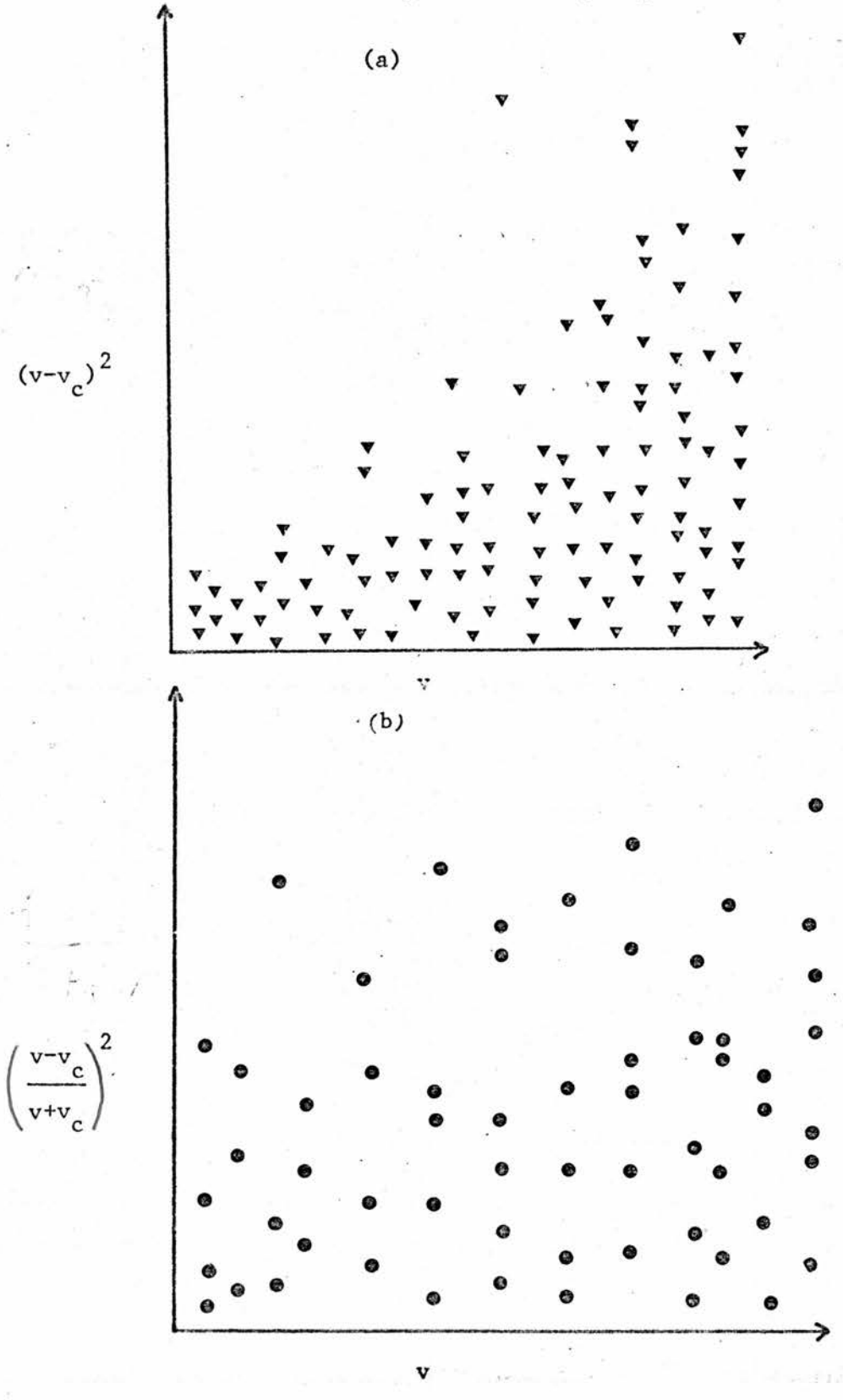
SIMPLEX is a good choice of optimization technique for situations where the equations are not well defined i.e. there is a lack of documentation of the relationships between data and experimental measurements, or where good initial estimates of the unknown variables cannot be obtained. These were problems often encountered during this study (see Chapters II and IV) and therefore SIMPLEX was the method of optimization employed. A computer program of this procedure (originally written in the Department of Statistics, University of Edinburgh, and adapted in this laboratory) was used. This handles a large variety of optimization problems well although it tends to be

rather slow where a large number of parameters are to be fitted (Davis & Ottaway, 1972). The program requires four subroutines to be adapted for each particular problem. These are the subroutines INSET, which is used for reading in the experimental data where manual input would be time-consuming; FINISH and FINAL, which are used for printing out information not printed out by the main program (e.g. the present values of the functions being optimized (V_{max}; K_m)), FINISH printing out at set points throughout the computation, FINAL printing out only after the minimum has been found; and COSTF in which the values of the sum of the squares deviations (called the COST) is calculated.

Since the SIMPLEX program was used to solve many different problems - the estimation of rate constants for the simulation study and the fitting of the experimental data to the rate equations for 2-oxoglutarate dehydrogenase - very many versions of these four subroutines were written. A few examples are shown in Appendix I. In the COSTF subroutine, the final value of the COST comprises all the 'deviations' between theoretical and experimental values of the relationships being optimized. The deviations may be weighted in a variety of ways. The standard statistical approach is to use the squared differences, since the variance ($\sum_{i=1}^{n} \frac{Dev^2}{n}$) is independent of the size of the observations. However it is generally accepted that this is not true for enzymic reaction velocity measurements (see also Fig. III.2,a), since the variance is not independent of velocity (Storer et al., 1975). There have been many suggestions for weighting so that kinetic parameters may be obtained more accurately. The weighting suggested by Ottaway (1973) is purely empirical:

$$\text{variance} = \left(\frac{v - \hat{v}}{v + \hat{v}} \right)^2$$

FIG.III.2. Graphs depicting the distributions of the deviations of the experimental data from the 2-oxoglutarate dehydrogenase studies.



where v is the measured initial velocity, $\bar{v}$ is the mean value of two duplicate measurements of v and v_c is the value calculated in the SIMPLEX optimization.

(a) represents the distribution of the squared differences.

(b) represents the distribution of the proportional deviations for mechanism 5 of FIG.IV.vii.7.

where v= measured velocity and $\hat{v}$ is the mean velocity at that point (replicate measurements are essential) and its virtue lies in the fact that it prevents distortion at high and low values of v especially during optimization procedures such as SIMPLEX. This approximates to: $\text{variance} = k.v^2$

i.e. constant relative error, and has been criticised by Storer et al. (1975) on the grounds that it implies that variance is completely proportional to velocity, which is not a general property of this type of experimental data. Figs. III.2 a,b show that in the results of the initial velocity studies with 2-oxoglutarate dehydrogenase reported in Chapter IV, there is an approximate proportion between variance and velocity, and in this case it was decided to use the criterion of Ottaway (1973) for weighting the deviations as the approximation is accurate enough with these data (see Fig.III.2 b) for the weighting to be valid (see also Askelöf et al., 1976).

When fitting the rate constants to the kinetic parameters the weighting employed was such that the deviations were adjusted to similar orders of magnitude for each rate constant. This avoided attempts by the program to fit the largest numerical values proportionately better, since these would contribute significantly more to the 'COST' value, although the percentage difference between experimental and calculated values might not be better or worse than those for the parameters of small numerical size being fitted at the same time (see Appendix I for example of this).

ii) SYMAP

The SYMAP program was developed by and is available from The Laboratory for Computer Graphics and Spatial Analysis, Harvard Centre for Environmental Design Studies, Graduate School of Design, Harvard University, Cambridge, Mass. 02138, U.S.A.

This program was used during the analysis of the 2-oxoglutarate dehydrogenase kinetic studies, to display visually the fit between the experimental and theoretical values of the initial velocities corresponding to the different substrate concentrations used (c.f. Ottaway & Apps, 1972). Since the output can only be represented in two dimensions, three maps for each fitting were produced, one for each of the three combinations of two of the substrates.

For the mapping, the 'height' or 'density' corresponded to the percentage differences between experimental and calculated velocities, and these were mapped by a choropleth distribution. ('Choropleth' is the geographers' term for a map that displays average values over an area, rather than one which displays point values at a boundary (isopleth or contour map).)

The program operates by taking the set of heights (% difference) corresponding to each set of coordinates (concentrations), and calculating the average height. Where heights are not available for the coordinates, these are calculated by examining the neighbouring known heights and an estimated height calculated from these and inserted. This gives a general distribution which is easier to assess visually for 'areas' of good and bad fit, than a set of isolated points of data. The three maps together can give a good overall impression of the fit of the data to the equation, better for instance than graphs which use only one substrate at a time as a coordinate.

The input of substrate concentrations was adjusted by scaling factors, i.e. CoA concentrations were multiplied by 10, and NAD^+ concentrations by 1.25, so that all three substrates were on the same scale for mapping, and the maps produced were therefore square in shape.

iii) The CHEK program

The method of operation of this program has been outlined previously (in Chapter II) in connection with the setting up of the simulation model.

The input for CHEK is in free format, although the program itself is written in Fortran. A description of the layout of the input file and examples of one of the programs used for the Cycle simulations are shown in Appendix II.

Once the equation deck had been set up, the running conditions for simulation are specified in the command deck. The commands which were most frequently used and which will be found in Appendix II, are briefly described here.

For the variables named the MAX commands specify the 'full scale value' against which the program for integrating the differential equations will assess accuracy specifications; that is an overall accuracy of, for example 0.001 is interpreted as meaning for each variable, an error bound of one thousandth of its 'full scale' value. If a negative value is entered, the value used for each named variable is initially the absolute value given, but is updated to follow the variable itself if its value either increases or decreases beyond the initial value thus enabling relative accuracy to be specified. This facility it useful for those chemical species which are products of a reaction, and whose concentration grows steadily throughout the simulation run, often starting at zero, or for those whose concentration becomes very small but nevertheless controls a reaction rate.

The GRAPH commands specify the conditions of graphical output; the letter represents the named variable and the value given is the maximum concentration represented on the graph. The commands NSTEP and DELTA specify the time over which the simulation is run; NSTEP

being the number of points of output on the graph and DELTA the time interval between them. Both these commands can be altered throughout the run so that transient features or transitons from one state to another can be studied in detail, whereas at a steady state there is no need to plot many points to follow the progress.

The BEGIN command informs the program that all necessary information for the simulation has been supplied and instructs the start of simulation. This command always refers to simulation from the initial conditions. The command RUN is used to continue the simulation for a further time span (denoted by new NSTEP and DELTA commands), starting off from the conditions existing at the end of a previous part of the simulation. It can only be used where no changes in conditions have been made i.e. the simulation was stopped only to supply information on the concentrations of the variables and reaction fluxes.

A complete list of the concentrations of the variables and the fluxes through the reactions can be printed at any point in time during the simulation by the use of the command LIST 2. Concentrations of the enzymes or the intermediates can be changed during the simulation by the use of the command: CONC 'var' X Y; where 'var' is the name of the variable being changed, X is the new concentration and Y the new MAX value. The rate constant of any reaction can be altered by the command: K I x ; where I is the number of the rate constant to be changed and x is its new value. Both the commands 'CONC' and 'K' must be followed by a BEGIN statement (not RUN). Should these changes be desired in conditions existing at the end of the first part of the simulation run, the command SAVE is entered before the 'CONC' or 'K' commands such that the order would be:

SAVE ;

CONC var X Y ;

K I x ;

BEGIN ;

and the simulation would proceed from the point at which it left off, changing only the values set by the CONC and/or K commands.

iv) Calculation of Rate Equations for 2-Oxoglutarate Dehydrogenase

Steady state rate equations for the different mechanisms proposed for 2-oxoglutarate dehydrogenase system were calculated in two ways, manually and by using the computer program of Silvestri & Zahner (1967).

To evaluate rate equations manually the method of King & Altmann, as described in Plowman (1972) was used. For the simpler mechanisms the equations were taken directly from Cleland (1963).

Initial rate equations were taken as the rate equations with product terms set to zero.

v) Computer Fitting of Initial Rate Data for 2-Oxoglutarate Dehydrogenase to Double Reciprocal Plots.

Initial velocity measurements for the 2-oxoglutarate dehydrogenase studies were fitted by an adaptation of Cleland's (1967) computer program (Gardiner & Ottaway, 1969). Input is in the form of the S and v measurements for one assay, and these are fitted by a least squares method to a rectangular hyperbola using initial estimates of 'K_m' and 'V_{max}' calculated within the program. Repeated iterations are carried out on the data until the best fit is found. If there is significant deviation from the hyperbolic function the data will not be fitted. A graph of the correspondence between experimental and theoretical fitting is printed out as a double reciprocal plot.

vi) Computer Programs - General Information

All computer programs used were stored in the E.M.A.S. (Edinburgh Multi Access System) on an ICL 4-75 computer. They were accessed via on-line teleprinter terminals situated in the Biochemistry Department (University of Edinburgh). Output was listed either directly on the terminal or by the lineprinter situated at the Edinburgh Regional Computing Centre. Early simulation studies were run on an IBM 370/158 machine using the CHEK program. Input for this was entered via card decks or through a file on E.M.A.S. The CHEK program was later put up on E.M.A.S. and required only minimal editing to adjust for a slight difference in character codes between the two machines.

E.M.A.S. operates on a time-sharing system and each program was limited to 2 minutes run time (CPU time) during the day. Longer programs were therefore run overnight. SYMAP and programs for fitting initial rate data to double reciprocal plots ran within 2 minutes CPU time. The time taken for SIMPLEX fittings was dependent on the problem. Most of the optimizations which involved fitting individual rate constants to values of kinetic constants for the enzymes which were used for Cycle simulations, ran within 2 minutes, whereas the fitting of the rate equations to initial velocity measurements for 2-oxoglutarate dehydrogenase took 10-30 minutes CPU time.
The run time for the program to calculate rate equations also varied with the size of the problem.

The CHEK program when mounted on the 370/158 computer would run both simulations of single enzymes or the entire Cycle in less than 1 minute CPU time. With the ICL 4-75 machine, single enzyme simulations ran within the 2 minute limit. Simulations of the Cycle took from 2-10 minutes depending on the size of the simulation, the conditions being examined, and the number of condition changes within a run.

For example, a simulation of the full equation deck for the Cycle ran to steady state (approx. 2000 secs of simulated time) in 28 seconds on the IBM 370/158 machine and 129 seconds on the ICL 4-75 machine.

vii) Calculation of Elasticities and Sensitivities

The definitions of Elasticity and Sensitivity were introduced by Kacser & Burns (1973). Sensitivity is a measure of the 'control strength' (c.f. Higgins, 1965b; Heinrich & Rapaport, 1974) of an enzyme and is defined as the fractional change in flux through a pathway caused by a fractional change in the enzyme concentration:

$$\text{SENSITIVITY} = \frac{\Delta \underline{f}/\underline{f}}{\Delta\{E\}/\{E\}}$$

where $\underline{f}$ is the flux through the pathway and $\{E\}$ is the enzyme concentration. The numerical value of the Sensitivity indicates the measure of control exerted by that enzyme, since Kacser & Burns showed that (for concentrations of enzymes much lower than their substrates) ΣS for the whole system = 1.

The Elasticity (effector strength) is defined as the fractional response in flux through a pathway to a fractional change in concentration of an effector - an effector being a metabolite (it may be a substrate or product) which interacts with more than one enzyme in the pathway. The Elasticity is a means of comparing the action of changing effector concentrations on the different enzymes to which the effector binds.

Evaluation of the Sensitivities using the simulation model was very simple. The simulation was run to steady state, then this was repeated with the concentration of one of the enzymes changed by 1%. This was carried out for each of the enzymes in turn. The theory of Kacser & Burns refers to the true values of Sensitivity and

Elasticity obtained by measuring the limiting value of $\frac{\Delta f}{f} \Big/ \frac{\Delta\{E\}}{\{E\}}$ i.e. $\frac{\partial f}{f} \Big/ \frac{\partial\{E\}}{\{E\}}$, however such infinitesimal changes cannot be achieved in reality or in simulation, but the true values can be approximated by making very small changes in {E}. The figure of 1% change was used for the simulation studies as the best compromise value. A smaller variation in {E} would be preferable (H. Kacser & J.A. Burns, personal communication), but, the flux changes would be difficult to detect under such conditions since the accuracy of integration of the CHEK program was probably of the order of 10^{-6} (J.M. Aitchison, personal communication).

The Elasticity values were calculated in two ways, a) for the enzymes within the Cycle and b) in isolation. In the former case the Cycle was simulated in steady state with 'dummy' reactions to hold the concentrations of all the metabolites at their steady state values except the effector under study which was varied by 1% of its steady state value. (The reasoning behind the use of the 1% value is also valid here.) The Elasticities for the isolated enzymes were evaluated by simulating each enzyme involved with the effector on its own. The enzyme was set to operate with the steady state flux and metabolite concentrations which were provided by the simulation of the complete Citric Acid Cycle at a steady state. The effector concentration was then varied, again by 1%.

It was found to be imperative in all these studies that the flux values are taken only when the system is truly in a steady state. In the simulation studies the criterion used was that the fluxes through each of the enzymes and the ancillary reactions were constant at the beginning and end of a simulation interval.

Section II. Chemical Methods

Preparation and Assay of 2-Oxoglutarate Dehydrogenase from Pig Heart

Materials Used

Coenzyme A, NAD^+ and 2-oxoglutarate were obtained from Boehringer & Soehne (Mannheim, W. Germany), cysteine hydrochloride from B.D.H. Ltd. (Poole, Dorset, U.K.). CoA was obtained as required and used fresh. Other chemicals used were of analytical grade. All solutions used for the initial velocity study except the buffer were made up fresh each day and stored in ice until used. All solutions used in the preparation of the 2-oxoglutarate dehydrogenase complex were stored and used at $4^{\circ}C$.

Protein estimations were carried out by the Lowry method.

The 2-oxoglutarate dehydrogenase complex was prepared from fresh pig heart muscle (from newly-slaughtered animals) using the method of Sanadi (1969). The concentrations of the final enzyme solutions were between 1.3 and 3.0 mg. protein/ml. with a specific activity of between 0.75 and 0.85 µmol NADH produced/minute/mg protein at $30^{\circ}C$. This specific activity is rather low compared to that quoted by Sanadi (1969) or Massey (1960). Attempts to purify the enzyme further were made using the methods of Massey (1960) and Severin & Gomazkova (1971). These were specifically designed to remove excess lipoyl dehydrogenase subunits which are a common 'contaminant' of preparations of the enzyme. The former method employs a calcium-phosphate cellulose column, the latter a Sephadex G-200 column. Both of these attempts were unsuccessful because the flow rate was too slow, and the enzyme lost most of its activity during chromatography at $4^{\circ}C$. Severin & Gomazkova (1971) suggested the loss of activity in this way was due to a separation of the subunits of the enzyme complex. Consequently it was decided to use the Sanadi preparation of the enzyme for the kinetic assays as

this gave measurable initial velocities. It was hoped that the presence of the extra lipolyl dehydrogenase protein (or other inactive protein) would not have any effect in the kinetic measurements.

The preparation was tested for pyruvate dehydrogenase activity, and for a requirement for added thiamine pyrophosphate and magnesium ions. All of these tests gave negative results.

The final enzyme solution was stored at $-4^{o}C$, and did not show any appreciable loss of activity while stored in the frozen state, although on thawing and refreezing activity did decrease. To avoid this, the final solution was stored in 1 ml aliquots, each aliquot being thawed and used within one day.

Initial velocity behaviour was examined using assay mixtures set up according to Fromm (1967). In this procedure the concentration of one of the substrates (S_x) is varied within an assay while the concentrations of the remaining two substrates (S_y & S_z) are held constant. For a set of assays with S_x as the variable substrate, the concentrations of S_y and S_z are both changed between assays, but the ratio of S_y:S_z is kept constant. For each set of assays, five concentrations of the variable substrate (S_x) and four concentrations of the 'fixed' substrates (S_y and S_z) were used and each measurement was made in duplicate. As each substrate in turn was made the 'variable' one (S_x) the total number of measurements in each run was $((5 \times 4) \times 2) \times 3 = 120$.

Assays were carried out at $30^{o}C$ using an S.P. 1800 recording spectrophotometer in which the temperature was regulated by a continuous flow of water which was thermostatically maintained at $30^{o}C$. Cuvettes containing assay mixtures were pre-incubated at $30^{o}C$ for approximately 5 minutes before initiating the reaction by the addition of 0.01 ml enzyme solution. CoA and NAD^{+} aliquots were added just prior to the pre-incubation stage in order to

minimise non-enzymic destruction. The assay mixtures all contained in 1 ml potassium phosphate buffer 0.08M (pH 7.2) and cysteine hydrochloride 0.4mM (pH 7.0-7.5). The concentration ranges of substrate were: 2-oxoglutarate (pH 7.2) 0.025-0.5 mM; CoA, 0.005-0.1 mM and NAD^+ (pH 7.2) 0.02-0.4 mM. The ratios of the fixed substrates (S_y:S_z) used for the three sets of assays were, CoA:NAD^+, 1:8; 2-oxoglutarate:CoA, 10:1; and 2-oxoglutarate:NAD^+, 5:4.

The progress of each reaction was followed for 2-3 minutes and the initial velocity taken as the linear part of the progress curve immediately after addition of enzyme. The length of the linear section of the curve was dependent on the substrate concentrations.

The activity of the enzyme was constantly monitored throughout the day, and any decrease in its activity, which was usually slight, was corrected for by adjusting the initial rate estimates. After these adjustments, the initial velocities were expressed in units of µmol NADH prod./min/mg protein. Results were plotted in double reciprocal form.

Some product inhibition studies were carried out with NADH as the inhibitor. Where NAD^+ was the variable substrate, inhibition with CoA and 2-oxoglutarate at both saturating and non-saturating concentrations was examined. Where CoA or 2-oxoglutarate was the variable substrate, the 'fixed' substrates were at saturating concentrations. For each study five concentrations of the variable substrate and four concentrations of the inhibitor (including zero) were used. Results were plotted in double reciprocal form and as Wilkinson plots ($\frac{S}{v}$ against S). The resulting patterns of plots were analysed for type of inhibition.

Calculation of Enzyme Concentrations for Simulation of the Citric Acid Cycle

The method of Srere (1968) was used to calculate enzyme concentrations within mitochondria for the enzymes aconitase, succinic dehydrogenase, and thiolase.

ACONITASE

The value for the turnover number was taken from Villafranca and Mildvan (1971) and the mitochondrial activity of the enzyme from Bachmann et al. (1966).

Turnover number = 13.5 mol citrate converted/sec/mol enzyme

= 810 " " " min " "

Tissue activity = 300×10^{-9} mol cit converted/min/mg mito prot.

$$\text{Enzyme Concentration} = \frac{300 \times 10^{-9} \text{ mol cit converted/min/mg mito prot.}}{810 \text{ mol cit converted/min/mol enzyme}}$$

$$= \frac{30}{81} \times 10^{-9} \text{ mol enz/mg mito prot.}$$

$$= \frac{\frac{30}{81} \times 10^{-9} \text{ mol enz/mg mito prot.}}{5 \times 10^{-6} \text{ kg mito/mg mito prot.}}$$

$$= 0.074 \times 10^{-3} \text{ mol enz/kg mitochondria.}$$

SUCCINIC DEHYDROGENASE

The turnover number is taken from Singer et al. (1973) and the tissue activity from Bachmann et al. (1966).

Turnover number = 18,000 mol succ. oxid/min/mol enz.

Tissue activity = 200×10^{-9} mol succ. oxid./min/mg mito prot.

$$\text{Enzyme Concentration} = \frac{200 \times 10^{-9} \text{ mol succ. oxid./min/mg mito prot.}}{18000 \text{ mol succ. oxid./min/mol enzyme}}$$

$$= \frac{1}{90} \times 10^{-9} \text{ mol enz/mg mito prot.}$$

$$= \frac{1/90 \times 10^{-9} \text{ mol enz/mg mito prot.}}{5 \times 10^{-6} \text{ kg mito/mg mito prot.}}$$

$$= 0.0023 \times 10^{-3} \text{ mol enz/kg mito.}$$

ACETYL CoA Acetyl Transferase

The turnover number for this enzyme was taken from Gehring and Lynen (1972) and the tissue activity from Middleton (1973).

Turnover number = 20,000 mol acetoacetyl CoA utilized/min/mol enz.

Tissue activity = 18.6×10^{-6} mol acetoacetylCoA utilized/min/gm wet wt tissue

= $18.6 \times 5 \times 10^{-6}$ mol acetoacetylCoA utilized/min/gm mitochondria

= $18.6 \times 5 \times 10^{-9} \times 10^{-3}$ mol acetoacetylCoA utilized/min/kg mitochondria

$$\text{Enzyme Concentration} = \frac{18.6 \times 5 \times 10^{-3} \text{ mol acetoacetylCoA utilized/min/kg mito.}}{20{,}000 \text{ mol acetoacetylCoA utilized/min/mol enz.}}$$

= 4.65×10^{-6} mol enz./kg mito.

= 0.00465 mmol enz/kg mitochondria.

APPENDIX I. Subroutines for the SIMPLEX procedure to fit Initial Rate Data (from Experimental Studies on 2-Oxoglutarate Dehydrogenase) directly to a Rate Equation describing a Semi-Random Mechanism: Subroutine INSET.

```
      SUBROUTINE INSET
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION ARRAY1(240),ARRAY2(240),ARRAY3(240),ARRAY(240)
      COMMON/CONST/ ARRAY1,ARRAY2,ARRAY3,ARRAY
      M=-1.0
      DO 103 N=1,60
      READ(5,101)A,B,C
  101 FORMAT(1H0,3F6.4)
      M=M+2
      J=M+1
      DO 102 I=M,J
      ARRAY1(I)=A
      ARRAY2(I)=B
      ARRAY3(I)=C
      READ(5,104)ARRAY(I)
  104 FORMAT(1H ,F7.6)
      IF(ARRAY(I).EQ.0.00000) GOTO90
      GOTO 102
   90 ARRAY1(I)=0.0000
      ARRAY2(I)=0.0000
      ARRAY3(I)=0.0000
  102 CONTINUE
  103 CONTINUE
      RETURN
      END
```

Subroutine 'COSTF'.

```
      SUBROUTINE COSTF(X,N,COST)
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION X(N),DEV(240),V(240),DIFF(240),PCENT(240),ARR(240)
      COMMON/CONST/ ARRAY1(240),ARRAY2(240),ARRAY3(240),ARRAY(240),DEV,
     1V,PCENT,C1,C2,C3,C4,C5,C6,C7,C8,C9,C10,C11,C12,C13,C14,SSQ
C     THIS IS AN ATTEMPT TO FIT THE EXPERIMENTAL DATA DIRECTLY
C     TO THE INITIAL RATE EQUATION FOR THE 1ST SEMI RANDOM MECHANISM
C     DEVS ARE CALC'D AS PROPORTIONAL DEVS-IE-(V-CALC.V)/V+CALC.V
C     MECHANISM STUDIES   17.12.75
      DO 100 J=1,N
      IF(X(J).GE.1.0E8) X(J)=1.0E8
  100 IF(X(J).LE.1.0E-8) X(J)=1.0E-8
      C1=X(1)
      C2=X(2)
      C3=X(3)
      C4=X(4)
      C5=X(5)
      C6=X(6)
      C7=X(7)
      C8=X(8)
      C9=X(9)
      C10=X(10)
      C11=X(11)
      C12=X(12)
      C13=X(13)
      C14=X(14)
      COST=0.0D0
      DO 101 I=1,120
      IF(ARRAY1(I).EQ.0.0000)GOTO102
      V(I)=(C1*ARRAY1(I)*ARRAY2(I)*ARRAY3(I)+C2*ARRAY1(I)*ARRAY2(I)
     1*ARRAY2(I)*ARRAY3(I)+C3*ARRAY1(I)*ARRAY2(I)*ARRAY3(I)*ARRAY3(I))
     2/(C4*ARRAY1(I)+C5*ARRAY1(I)*ARRAY2(I)+C6*ARRAY1(I)*ARRAY3(I)
     3+C7*ARRAY1(I)*ARRAY2(I)*ARRAY2(I)+C8*ARRAY1(I)*ARRAY3(I)*ARRAY3
     4(I)+C9*ARRAY2(I)*ARRAY3(I)+C10*ARRAY1(I)*ARRAY2(I)*ARRAY3(I)+C11
     5*ARRAY1(I)*ARRAY2(I)*ARRAY3(I)*ARRAY3(I)+C12*ARRAY1(I)*ARRAY2(I)
     6*ARRAY2(I)*ARRAY3(I)+C13*ARRAY2(I)*ARRAY3(I)*ARRAY3(I)+C14*
     7ARRAY2(I)*ARRAY2(I)*ARRAY3(I))
      ARR(I)=ARRAY(I)/60.0
      DIFF(I)=(ARR(I)-V(I))/(ARR(I)+V(I))
      DEV(I)=DIFF(I)*DIFF(I)
      PCENT(I)=((ARR(I)-V(I))/ARR(I))*100.0
      COST=COST+DEV(I)
      SSQ=COST
      GOTO 101
  102 V(I)=0.0
      DEV(I)=0.0
      PCENT(I)=0.0
  101 CONTINUE
      RETURN
      END
```

Subroutines FINISH and FINAL.

```
      SUBROUTINE FINISH
      IMPLICIT REAL*8(A-H,O-Z)
      COMMON/CONST/ ARRAY1(240),ARRAY2(240),ARRAY3(240),ARRAY(240),
     1DEVV(240),V(240),PCENT(240),C1,C2,C3,C4,C5,C6,C7,C8,C9,C10,C11,
     2C12,C13,C14,COST
      WRITE(6,201)
201   FORMAT(1H0,'    C1          C2          C3          C4          C5
     1     C6          C7    ')
      WRITE(6,202)C1,C2,C3,C4,C5,C6,C7
202   FORMAT(1H0,6(F8.3,1X,)F8.3,/)
      WRITE(6,203)
203   FORMAT(1H0,'    C8          C9          C10         C11         C12         C13
     1       C14    ')
      WRITE(6,204)C8,C9,C10,C11,C12,C13,C14
204   FORMAT(1H0,6(F8.3,1X,)F8.3)
      WRITE(6,200)COST
 200  FORMAT(//,'THE COST IS =',E15.7)
      RETURN
      END

      SUBROUTINE FINAL
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION RECIP(240),REC(240)
      COMMON/CONST/ ARRAY1(240),ARRAY2(240),ARRAY3(240),ARRAY(240),
     1DEV(240),V(240),PCENT(240)
      WRITE(6,206)
 206  FORMAT(1H0,'  K   CONC.A',2X,'CONC.B',2X,'CONC.C',3X,'INT.V',5X,
     1'CALC.V',7X,'DEV',6X,'%',8X,'1/INT.V',7X,'1/CALC.V',//)
      DO 207 K=1,120
      IF(ARRAY(K).EQ.0.0000)GOTO111
      IF(V(K).EQ.0.0000)GOTO112
      RECIP(K)=1.0/ARRAY(K)
      REC(K)=1.0/(V(K)*60.0)
      GOTO113
 111  RECIP(K)=0.0
 112  REC(K)=0.0
 113  WRITE(6,205)K,ARRAY1(K),ARRAY2(K),ARRAY3(K),ARRAY(K),V(K),DEV(K)
     1,PCENT(K),RECIP(K),REC(K)
 205  FORMAT(1H0,I3,1X,F6.4,2X,F6.4,2X,F6.4,2X,F8.6,2X,F10.8,2X,E10.5,
     12X,F6.2,2X,E12.5,2X,E12.5)
 207  CONTINUE
      RETURN
      END
```

'COSTF' subroutine for SIMPLEX procedure to find rate constants for 2-Oxoglutarate Dehydrogenase using the published data.

```
      SUBROUTINE COSTF(X,N,COST)
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION X(N)
      COMMON/CONST/ VMAX, EQK, AKM, COKM, DKM, SKM
      DO 110 J=1,N
      IF(X(J).LT.1.0D-6) X(J)=1.0D-6
110   IF(X(J).GT.1.0D8) X(J)=1.0D8
      C1=X(1)
      C2=X(2)
      C3=X(3)
      C4=X(4)
      C5=X(5)
      C6=X(6)
      C7=X(7)
      C8=X(8)
      C9=X(9)
      C10=X(10)
      COST=0.0D0
C     THIS IS THE VMAX FOR THE FORWARD REACTION
      VMAX=(C3*C7)/(C3+C7)
      DEV=(570.0-VMAX)/10.0
      DEV=DEV*DEV
      COST=COST+DEV
C      THIS IS THE KM FOR OXOGLUTARATE
      AKM=(C3*C7)/(C1*(C3+C7))
      DEV=(0.013-AKM)*10000.0
      DEV=DEV*DEV
      COST=COST+DEV
C      THIS IS THE KM FOR COA
      COKM=(C7*(C3+C4))/(C5*(C3+C7))
      DEV=(0.0001-COKM)*100000.0
      DEV=DEV*DEV
      COST=COST+DEV
C      THIS IS THE KM FOR NAD
      DKM=(C3*(C7+C8))/(C9*(C3+C7))
      DEV=(0.0045-DKM)*10000.0
      DEV=DEV*DEV
      COST=COST+DEV
C      THIS IS THE KM FOR SUCCINYLCOA
      SKM=(C4*(C7+C8))/(C6*(C4+C8))
      DEV=(0.03-SKM)*1000.0
      DEV=DEV*DEV
      COST=COST+DEV
C      THIS IS THE EQUILIBRIUM CONSTANT
      EQK=(C1*C3*C5*C7*C9)/(C2*C4*C6*C8*C10)
      DEV=(7.5D5-EQK)/10000.0
      DEV=DEV*DEV
      COST=COST+DEV
      RETURN
      END
```

Subroutines FINISH, FINAL and INSET for SIMPLEX procedure to find rate constants for 2-Oxoglutarate Dehydrogenase using the published data.

```
      SUBROUTINE FINISH
      IMPLICIT REAL*8(A-H,O-Z)
      COMMON/CONST/ VMAX, EQK, AKM, COKM, DKM, SKM
      WRITE(6,91)
91    FORMAT(1H0,'   VMAX       EQK       DKM       AKM       COKM       SKM'/
     *' 570.0    7.5D5    0.0045    0.013   0.0001      0.03')
      WRITE(6,92) VMAX, EQK, DKM, AKM, COKM, SKM
92    FORMAT(//,F8.2,D8.1,F8.4,F8.4,F8.5,F8.4)
      RETURN
      END

      SUBROUTINE INSET
      RETURN
      END

      SUBROUTINE FINAL
      RETURN
      END
```

Subroutine 'COSTF' for SIMPLEX procedure on kinetic data for Isocitrate Dehydrogenase.

```
      SUBROUTINE COSTF(X,N,COST)
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION X(N)
      COMMON/CONST/ VMAXF,EQK,XKMNAD,XKMIC,XKNADH,XKICO2,XKIAOG
      DO 110 J=1,N
      IF(X(J).LT.1.0D-5) X(J)=1.0D-5
  110 IF(X(J).GT.1.0D6) X(J)=1.0D6
      C1=X(1)
      C2=X(2)
      C3=X(3)
      C4=X(4)
      C5=X(5)
      C6=X(6)
      C7=X(7)
      C8=X(8)
      C9=X(9)
      C10=X(10)
      COST=0.0D0
C     THIS IS THE VMAX FOR THE FORWARD REACTION
      VMAXF=(C5*C7*C9)/(C5*C7+C7*C9+C5*C9)
      DEV=(133.0-VMAXF)
      DEV=DEV*DEV
      COST=COST+DEV
C     THIS IS THE KM FOR NAD
      XKMNAD=(C5*C7*C9)/(C1*(C5*C7+C7*C9+C5*C9))
      DEV=(0.08-XKMNAD)*100.0
      DEV=DEV*DEV
      COST=COST+DEV
C     THIS IS THE KM FOR ISOCITRATE
      XKMIC=(C7*C9*(C4+C5))/(C3*(C5*C7+C7*C9+C5*C9))
      DEV=(0.14-XKMIC)*100.0
      DEV=DEV*DEV
      COST=COST+DEV
C      THIS IS THE KI FOR NADH
      XKNADH=C9/C10
      DEV=(0.04-XKNADH)*100.0
      DEV=DEV*DEV
      COST=COST+DEV
C     THIS IS THE EQUILIBRIUM CONSTANT
      EQK=(C1*C3*C5*C7*C9)/(C2*C4*C6*C8*C10)
      DEV=(855.0-EQK)
      DEV=DEV*DEV
      COST=COST+DEV
C     THESE ARE THE DUMMY VALUES OF KI FOR CO2 AND OXOGLUTARATE
      XKICO2=C5/C6
      DEV=(1.0E3-XKICO2)/(1.0E3+XKICO2)
      DEV=DEV*DEV
      COST=COST+DEV
      XKIAOG=C7/C8
      DEV=(1.0E3-XKIAOG)/(1.0E3+XKIAOG)
      DEV=DEV*DEV
      COST=COST+DEV
      RETURN
      END
```

APPENDIX II. Annotated Example of an Input file for the CHEK program.

******* THIS IS THE INPUT FILE ********

```
* ; DEMONSTRATION OF ENZYME SIMULATOR PROGRAM                    !
* ; SIMULATION OF  FUMARASE AND MALATE DEHYDROGENASE             !----- COMMENTS
* ; FEBRUARY 1976    USING CHEK VERSION OF PROGRAM               !
** ;                                                            !

  CONCENTRATIONS          RATE CONSTANTS
    /       /               /       /
   /       /               /       /
  /       /               /       /                            !
0.001 E + 1.0 FUM = EFUM  / 0.11E+8 / 0.27E+5  ;                !
EFUM = EMAL / 0.23E+4 / 0.17E+4  ;                              !
EMAL = E + MAL /  0.46E+5 / 0.5E+7  ;                           !
0.002 MDH + 1.0 NAD = MDHNAD  /  5.5E2  /  5.7E2   ;           !
MDHNAD + MAL = MDHXY   /  2.0E3  /  3.3E4   ;                   !----- EQUATION DECK
MDHXY = MDHNADH + OAA   /  8.3E3  /  1.7E4   ;                  !
MDHNADH = MDH + NADH   /  1.7E2  /  3.3E4   ;                   !
NADH = NAD   /  10.   ;                                         !
** ;                                                           !

MAX -0.001 E EFUM EMAL   ;                                      !
MAX -1.0 FUM MAL NAD NADH OAA   ;                               !
MAX -0.001 MDH MDHNAD MDHXY MDHNADH   ;                         !
GRAPH OAA 0.5 0   ;                                             !
GRAPH FUM 1.0 F  ;                                              !
GRAPH MAL 1.0 M  ;                                              !
NSTEP  40  ;                                                    !
DELTA  0.05   ;                                                 !----- COMMAND DECK
BEGIN  ;                                                        !
LIST  2  ;                                                      !
NSTEP  50   ;                                                   !
DELTA  0.1   ;                                                  !
RUN   ;                                                         !
LIST  2  ;                                                      !
STOP   ;                                                        !
```

Example of an Input File for the CHEK program.

```
* ; SIMULATION OF THE T.C.A. CYCLE 21/10/74
* ; STEADY STATE SIMULATION
** ;
0.01 ST + 0.22E-2 SUCOA = STSUCOA / 370. / 11.4 ;
STSUCOA + 0.7211 P = ST*SUP / 43.8 / 65.2 ;
ST*SUP = ST*P + SUCC / 66.7 / 24.0 ;
ST*P + 0.9786E-1 GDP = ST*GTP / 2268. / 27.2 ;
ST*GTP = ST* + 0.1504E-2 GTP / 1217. / 40804. ;
ST* = ST + COA / 13.4 / 547.4 ;
0.23E-2 SDH + 0.289E-1 SUCC = SDHSUCC / 36. / 2.2 ;
SDHSUCC + 0.5 AOX = SDHFUM + 0.5 ARED / 7.7 ;
SDHFUM = SDH + FUM / 130. / 69. ;
0.8E-2 FASE + 0.986E-1 FUM = FASEFUM / 0.11E8 / 0.27E5 ;
FASEFUM = FASEMAL / 0.23E4 / 0.17E4 ;
FASEMAL = FASE + MAL / 0.46E5 / 0.5E7 ;
0.07 MDH + 0.2372 NAD = MDHNAD / 5.5E2 / 5.7E2 ;
MDHNAD + 0.5 MAL = MDHXY / 2.0E3 / 3.3E4 ;
MDHXY = MDHNADH + OAA / 8.3E3 / 1.7E4 ;
MDHNADH = MDH + 0.2674 NADH / 1.7E2 / 3.3E4 ;
1.0 PREC1 = 1.0 PREC1 + 0.33E-1 ACCOA / 33. / 1E3 ;
0.026 CS + 0.6342E-4 OAA = CSOAA / 1E3 / 5. ;
CS + ACCOA = CSACCOA / 1E3 / 4.5 ;
CS + SUCOA = CSI / 1E3 / 130. ;
CSOAA + ACCOA = CSOAAACCOA / 1E3 / 4.5 ;
CSACCOA + OAA = CSOAAACCOA / 1E3 / 5. ;
CSOAA + SUCOA = CSI* / 1E3 / 130. ;
CSOAAACCOA = CSCITCOA / 283. / 0.2 ;
CSCITCOA = CSCOA + CIT / 300. / 1E3 ;
CSCITCOA = CSCIT + COA / 30. / 1E3 ;
CSCOA = CS + COA / 30. / 1E3 ;
CSCIT = CS + CIT / 300. / 1E3 ;
0.074 AC + 0.2089E-1 CIT = ACCIT / 550. / 1330. ;
ACCIT = ACX / 95. / 65. ;
ACX = ACA / 1210. / 9200. ;
ACA = AC + A / 360. / 750. ;
ACX = ACIC / 2900. / 2300. ;
ACIC = AC + IC / 43. / 510. ;
0.092 ID + NAD = IDNAD / 1755. / 4874. ;
IDNAD + 0.1278E-2 IC = IDX / 1396. / 476. ;
IDX = IDY + CO2 / 1181. / 3.8 ;
IDY = IDNADH + AOG / 1660. / 26. ;
IDNADH = ID + NADH / 165. / 4082. ;
ID + ATP = IDI / 1E3 / 150. ;
0.006 KGDH1 + 0.8011E-5 AOG = KGDH2 + CO2 / 4.4E4 / 1.24E5 ;
KGDH2 = KGDH3 / 1.73E4 / 66.0 ;
KGDH3 + COA = KGDH4 + SUCOA / 5.7E6 / 1.12E4 ;
KGDH4 = KGDH5 / 590. / 62. ;
KGDH5 + NAD = KGDH1 + NADH / 1.4E5 / 8.35E4 ;
CO2 = BICARB / 100. ;
NADH = NAD / 4700. / 5300. ;
0.711E-1 ATP = 4.633 ADP + P / 470. / 10. ;
ADP + GTP = ATP + GDP / 100. / 100. ;
AOX = ARED / 100. / 100. ;
1.0 REM + 0.066 COA = 1.0 REM / 1000. / 66. ;
** ;
```

```
MAX -0.2234E-2 SUCOA  ;
MAX -0.9786E-1 GDP  ;
MAX -0.1504E-2 GTP  ;
MAX -0.289E-1 SUCC  ;
MAX -0.5 AOX ARED  ;
MAX -0.986E-1 FUM  ;
MAX -0.5 MAL  ;
MAX -0.2372 NAD  ;
MAX -0.2674 NADH  ;
MAX -0.033 ACCOA  ;
MAX -0.6342E-4 OAA  ;
MAX -0.2089E-1  CIT  ;
MAX -0.1278E-2 IC  ;
MAX -0.8071E-5 AOG  ;
MAX -0.066 COA  ;
MAX -0.01 ST STSUCOA ST*SUP ST*P ST*GTP ST*  ;
MAX -0.23E-2 SDH SDHSUCC SDHFUM  ;
MAX -0.8E-2 FASE FASEFUM FASEMAL  ;
MAX -0.07 MDH MDHNADH MDHNAD MDHXY  ;
MAX -1.0 PREC1 REM  ;
MAX -0.026 CS CSOAA CSOAAACCOA CSACCOA CSI CSCITCOA CSI*  ;
MAX -0.026 CSCIT CSCOA  ;
MAX -0.074 AC ACCIT ACX ACA ACIC  ;
MAX -0.001 A  ;
MAX -0.092 ID IDNAD IDX IDY IDNADH IDI  ;
MAX -0.006 KGDH1 KGDH2 KGDH3 KGDH4 KGDH5  ;
MAX -0.711E-1 ATP  ;
MAX -4.633 ADP  ;
MAX -0.1E-3 CO2 BICARB  ;
GRAPH CIT 0.3 C  ;
GRAPH MAL 0.7 M  ;
GRAPH OAA 0.0001  O  ;
GRAPH IC 0.005  I  ;
GRAPH  AOG 0.5E-4  A  ;
GRAPH SUCOA 0.01  S  ;
NSTEP 50  ;
DELTA 3.0  ;
BEGIN  ;
LIST  2  ;
NSTEP 50  ;
DELTA 3.0  ;
RUN  ;
LIST  2  ;
NSTEP 50  ;
DELTA 3.0  ;
RUN  ;
LIST 2  ;
NSTEP  50  ;
DELTA  3.0  ;
RUN  ;
LIST  2  ;
NSTEP  50  ;
DELTA 3.0  ;
RUN  ;
LIST  2  ;
STOP  ;
```

CHAPTER IV

RESULTS AND DISCUSSION

Section i. Control Features of the Citric Acid Cycle Enzymes

The simulation was run to a steady state using the equation deck for the Citric Acid Cycle set up as detailed in Chapter II. In this simulation the concentrations of acetylCoA and free CoA, and the ratios of NAD^+:NADH, ATP:ADP, GTP:GDP and Aox:Ared were all held constant using the equations given in Chapter II (Fig. II.21). The concentrations of the Cycle intermediates were allowed to find their own steady state values within the simulation, and these are shown in Table IV.i.1. Steady state was achieved within 750 seconds of simulated time (the output from this run is shown in Appendix III). The starting values for the concentrations of the intermediates were taken from preliminary simulations which used the concentrations of intermediates given by Williamson & Corkey (1969) as starting values. The steady state concentrations of intermediates differed markedly from the data of Williamson & Corkey. This was only to be expected since the data of these authors referred to whole heart tissue perfused with glucose and insulin, and may not reflect mitochondrial contents very accurately.

Since in this simulated system the conditions were very strictly controlled i.e. the ratios of NAD^+:NADH, acetylCoA:CoA, ATP:ADP, GTP:GDP and Aox:Ared were not allowed to vary, the inherent control parameters of the enzymes of the Cycle could be investigated without having to allow for the effects of the rate of acetylCoA input or of regeneration of NAD^+ by the Electron Transport Chain.

The first study was an examination of the Sensitivities of the Cycle enzymes (for a definition of Sensitivity see Chapter III). Sensitivities of each of the enzymes of the Cycle were measured as explained in Chapter III and these are shown in Table IV.i.2.

TABLE IV.i.1. Concentrations of Intermediates and Cofactors of the Citric Acid Cycle at Steady State.

INTERMEDIATE/COFACTOR	CONCENTRATION (mmol/kg mito.)
Citrate	0.2179×10^{-1}
cis-Aconitate	0.8361×10^{-3}
Isocitrate	0.1337×10^{-2}
2-Oxoglutarate	0.7907×10^{-5}
SuccinylCoA	0.2196×10^{-2}
Succinate	0.2768×10^{-1}
Fumarate	0.9656×10^{-1}
Malate	0.4896
Oxalacetate	0.6210×10^{-4}
AcetylCoA	0.3300×10^{-1}
NAD^+	0.1719
NADH	0.1939
ATP	0.7035×10^{-1}
ADP	4.627
GTP	0.1486×10^{-2}
GDP	0.9753×10^{-1}
free CoA	0.6600×10^{-1}
$'A'_{ox}$	0.5
$'A'_{red}$	0.5
P_i	0.7146

Simulation of the Citric Acid Cycle with fixed ratios of NAD^+:NADH, ATP:ADP, GTP:GDP, AcetylCoA:free CoA. Cycle flux at Steady state is 0.1197×10^{-2} mmol/sec/kg mito.

TABLE IV.i.2. Sensitivities of Citric Acid Cycle Enzymes.

ENZYME	SENSITIVITY
Citrate Synthase	0.835
Aconitase	0.0
Isocitrate Dehydrogenase	0.083
2-Oxoglutarate Dehydrogenase	0.0
Succinyl Thiokinase	0.0
Succinic Dehydrogenase	0.083
Fumarase	0.0
Malate Dehydrogenase	0.0
	Σ1.001

Ratios of NAD^+:NADH, acetylCoA:free CoA, GTP:GDP, ATP:ADP are all held constant.

Sensitivity values measured by running the simulation to a steady state noting the flux. This is repeated with the concentration of one of the enzymes increased by 1% and the flux at steady state noted and used as described in Chapter III to calculate Sensitivity. This is repeated with each enzyme in turn.

Citrate synthase exhibited by far the greatest control over the Cycle flux with isocitrate dehydrogenase and succinic dehydrogenase showing minor Sensitivities. None of the other enzymes displayed any regulatory properties under these conditions.

These results support the proposal by Krebs & Lowenstein (1960) that, of the Cycle enzymes, the initiating enzyme of the pathway. citrate synthase, is the most important in the control of the Cycle flux. This conclusion does not agree with the view of Rolleston (1972) that citrate synthase behaves as an 'equilibrium' enzyme. There was very little evidence in this study to indicate that isocitrate dehydrogenase is an important Cycle regulator.

A preliminary examination of the effect of the NAD^+:NADH ratio in the Cycle operation was carried out by halving the ratio and running the simulation to a new steady state. The results of this study are shown in Table IV.i.3.

There were marked changes in both the flux and in the concentrations of intermediates at the new steady state, indicating that, as expected, the NAD^+:NADH ratio has multiple effects on the function of the Cycle. To examine this effect in more detail, the Elasticities of the Cycle enzymes towards NADH were measured (the term Elasticity is defined in Chapter III). The results of these studies are shown in Tables IV.i.4 and IV.i.5. Elasticities were measured with the NADH concentration at the value used in the steady state simulations and at five times this value. In both instances the NAD^+ concentration was held at its steady state level. As explained in Chapter III, the Elasticity values were measured both within the Cycle and by simulating the isolated enzymes. The enzymes which do not have NADH as a product would have been expected to have zero Elasticity towards this effector and where the Elasticities were measured within the Cycle simulation they were indeed very small with the exception of fumarase.

TABLE VI.i.3. Effects of Varying the NAD^+:NADH Ratio in the Citric Acid Cycle Simulation.

PARAMETER	Steady state concentrations (mmol/kg mito.) 'NORMAL'	$\frac{1}{2}\{NAD^+:NADH\}$
Citrate	0.218×10^{-1}	0.208×10^{-1}
Isocitrate	0.134×10^{-2}	0.130×10^{-2}
2-Oxoglutarate	0.791×10^{-5}	0.582×10^{-5}
SuccinylCoA	0.220×10^{-2}	0.112×10^{-2}
Succinate	0.277×10^{-1}	0.133×10^{-1}
Fumarate	0.966×10^{-1}	0.994×10^{-1}
Malate	0.490	0.504
Oxalacetate	0.621×10^{-4}	0.320×10^{-4}
NAD^+:NADH	0.887	0.4435
AcetylCoA:freeCoA	0.5	0.5
Cycle flux (μmoles/sec/Kg mito.)	1.20	0.62

'Normal' indicates the conditions are as in the previous situations (Tables IV.i.1 and 2).

TABLE IV.i.4. Elasticity of Citric Acid Cycle Enzymes Towards NADH.

ENZYME	APPARENT ELASTICITY	ABSOLUTE ELASTICITY
Citrate Synthase	-0.41	
Aconitase	-0.02	
Isocitrate Dehydrogenase	-0.76	-0.82
2-Oxoglutarate Dehydrogenase	-0.31	-0.37
Succinyl Thiokinase	0.0	
Succinic Dehydrogenase	0.0	
Fumarase	-0.23	
Malate Dehydrogenase	-21.7	-21.5

Flux at steady state is 1.2 mol/sec/kg mito. All conditions are as for Table IV.i.1. (NADH at 0.1939 mmol/kg mito.)

TABLE IV.i.5. Elasticity of Citric Acid Cycle Enzymes towards NADH.

ENZYME	APPARENT ELASTICITY	ABSOLUTE ELASTICITY
Citrate Synthase	-0.08	
Aconitase	-0.15	
Isocitrate Dehydrogenase	-0.92	-0.94
2-Oxoglutarate Dehydrogenase	-0.84	-0.81
Succinyl Thiokinase	0.0	
Succinate Dehydrogenase	0.0	
Fumarase	-5.24	
Malate Dehydrogenase	-33.4	-36.5

Flux at steady state is 0.25 mol/sec/kg mito. NADH concentration is 5 times 'normal' (0.9695mmol/kg mito.), all other conditions are as in previous situations.

These finite Elasticity values arise because there is a slight unbalancing of the steady state intermediate concentrations, as a result of the 'dummy' reactions which hold these concentrations at their steady-state values. The 'dummy' reactions provide a limitless capacity for supply/removal of the intermediates so that some of the enzyme reactions proceed at a much faster rate than the steady state Cycle flux. Thus although the concentrations are kept at steady-state values, the rate of production or removal by the dummy supplier enzymes is not necessarily the steady-state rate. This 'unbalances' the interactions between the enzymes, and the Elasticity of one enzyme towards NADH can upset a neighbouring reaction slightly. It was noticeable that this effect was only displayed by the enzymes around malate dehydrogenase, which is the enzyme exhibiting by far the largest Elasticity towards NADH. The fact that the Elasticities for the three NAD^+-linked dehydrogenases in isolation are very similar to those measured within the Cycle, implies that the Elasticities of the other enzymes, and the effects of the dummy reactions are of little or no consequence to the conclusions which were drawn from this study.

This work was very illuminating in another way. The steady-state flux, after increasing NADH concentration by a factor of five, decreased by a factor of approximately five. Since malate dehydrogenase shows by far the greatest Elasticity towards NADH, the Cycle flux must be heavily dependent on the concentration of oxalacetate. This effect is not determined by the rate of production of oxalacetate through malate dehydrogenase but is an effect of the equilibrium distribution of the substrates and products of this reaction. This conclusion can be deduced from the previous studies a) that the Sensitivity of malate dehydrogenase is zero (i.e. the ACTIVITY of the

enzyme has no control over the Cycle flux) and b) that halving the NAD^+:NADH ratio elicits no change in the equilibrium through malate dehydrogenase, i.e. {malate} x {NADH}/{oxalacetate} x {NAD^+} remains constant at 0.7×10^4 (see Table IV.i.3). This is rather a good illustration of the point made by Krebs (1969) as to the importance of 'equilibrium' enzymes - those enzymic reactions whose net flux is very much smaller than either forward or backward fluxes - with respect to their influence on substrate concentrations especially where a substrate/product might subsequently be involved in a 'pacemaker' reaction.

The Elasticity studies also imply that NADH produced within the first half of the Cycle by the isocitrate and 2-oxoglutarate dehydrogenases elicits its inhibitory effect on the Cycle flux by altering the equilibrium of malate dehydrogenase.Changing the concentration of NADH within these first two reactions, without changing it with respect to malate dehydrogenase, produces very little change in the Cycle flux. This can be seen as a mechanism by which reactions within the Cycle can prevent further entry of substrate acetylCoA into the Cycle by 'feed-forward' inhibition, acting through malate dehydrogenase, to reduce the concentration of oxalacetate, a much more sensitive regulator than inhibition of either the isocitrate or 2-oxoglutarate dehydrogenases themselves.
It is worth noting at this point that this conclusion remains valid whatever the mechanism for 2-oxoglutarate dehydrogenase (see Section viii. of this Chapter for fuller details).

Section ii. Oxalacetate Studies

It should be emphasised that in all discussions in this Chapter, the term 'concentration' of a metabolite refers to the free concentration. The concentrations of any enzyme-bound metabolites appear as separate entities in the CHEK program. With respect to the study of the Elasticity of the Cycle enzymes towards NADH discussed in the previous section, the equilibrium of malate dehydrogenase was calculated from the free concentrations of the substrates and products, in all situations.

Although in the simulations a substantial proportion of the oxalacetate is bound to enzymes (computation showed that this was ca. 30% of the total), and changes in the fraction bound did occur when the levels of NADH and malate dehydrogenase were altered, these changes had no effect on the Cycle flux (cf. section iv. of this Chapter where malate dehydrogenase shows a negative Sensitivity). Since malate concentration is approximately 10^4 times higher than oxalacetate, any decrease in the concentration of oxalacetate caused by increased binding is replenished by malate through a very rapid equilibrium of the malate dehydrogenase reactants.

However, the increase in bound oxalacetate in the situations described in the previous section was relatively small. In mitochondria there are very many binding sites for oxalacetate (e.g. succinic dehydrogenase (Zeilemaker et al., 1969b) and a number of subsidiary pathways utilising oxalacetate other than the Citric Acid Cycle). It has been suggested that such a 'removal' of free oxalacetate will have profound effects of the Citric Acid Cycle flux (Sols and Marco, 1970). To investigate this proposal, a short simulation study was carried out in which a dummy reaction simulating a sequestration of oxalacetate was included in the equation deck. This dummy reactant binds oxalacetate to form a 'dead-end' complex, i.e. it removes oxalacetate from the system:

0.4106E-1 SEQ + OAA $\rightleftharpoons$ SEQOAA / 590.0 / 1.23.

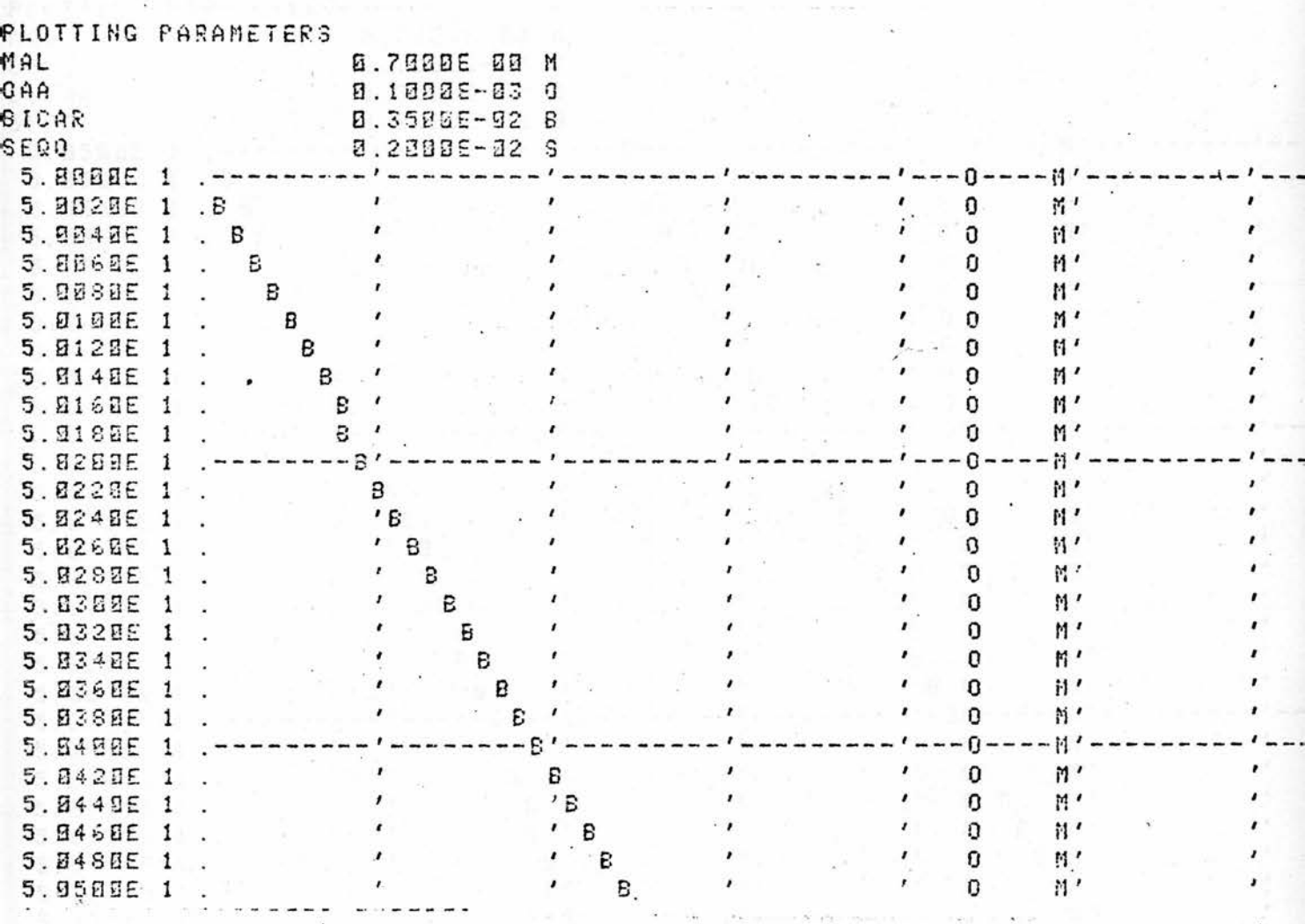
The rate constants for this reaction were set such that the amount of oxalacetate bound would be greater than the total free oxalacetate existing at steady state under the usual (previous) simulation conditions.

The simulation was run for a short time interval without this reaction included until a steady state was established, and then the sequestration reaction was inserted.

Graphical output of the results of this study are shown in Figs. IV.ii.1 and 2. Fig. IV.ii.1 shows the situation before, and Fig. IV.ii.2 after, including the oxalacetate sequestration reaction. It is clear from these results that removal of oxalacetate even by a very sudden, rapid and large sequestration has very little effect on the Cycle flux. The rate of CO_2 production (B on the graph) does not decrease appreciably, the steady-state level of oxalacetate drops only slightly (the initial larger fall in concentration lasts only for ca. 0.25 sec of simulated time), and the equilibrium of malate dehydrogenase very rapidly returns to its previous value.

This evidence invalidates suggestions of previous workers that sequestration of oxalacetate may have profound consequences on the flux through the Citric Acid Cycle. The fact that removal of oxalacetate is not important is due to two factors, a) the 'equilibrium' reaction of malate dehydrogenase and b) the high steady state concentration of malate. Malate serves as a latent pool of oxalacetate which can immediately restore its concentration should it fall for any reason. This is an extremely important function of malate dehydrogenase, that could not be performed if it were other than an 'equilibrium' enzyme. It is essential to stress that this assertion does not contradict in any way the conclusion drawn at the end of the previous section, namely that the steady-state concentration of oxalacetate is primarily determined

FIG.IV.ii.1. Simulation of the citric acid cycle and oxalacetate sequestration.



Graphical output from the simulation before oxalacetate sequestration is inserted.

where time (in seconds) is represented on the y-axis. The x-axis represents concentrations of malate (M) up to 0.7mM; oxalacetate (o) up to 0.1×10^{-3}mM; bicarbonate (B) up to 0.35×10^{-2}mM; and the 'sequestering agent' (S) up to 0.2×10^{-2}mM.

by the NAD/NADH ratio. Both this and the malate/oxalacetate ratio are dynamic. The crucial importance of this reaction to the efficient operation of the Citric Acid Cycle will be more fully discussed in Chapter V.

Section iii. Control of the Cycle by the Rate of Supply of AcetylCoA.

One further study with the 'minimal' Cycle model (that used in Section i.) was made, to test the effect of varying the input of acetylCoA to the Cycle. The acetylCoA concentration was doubled and then tripled to 0.066 and 0.1 mmol/kg mitochondria, respectively. All other parameters and variables were set as for previous simulations (NAD^+:NADH at the usual value). The results are shown in Table IV.iii.1. They show that doubling the concentration of acetylCoA increased the flux through the Cycle, but only by about 10%. As the concentration of acetylCoA was increased further, the increase in flux was still less. The major effect on the Cycle appeared to be a decrease in the concentrations of fumarate, malate and oxalacetate, with an increase in the levels of all the other intermediates. It is striking that the Cycle flux is not as responsive to changes in the acetylCoA concentration as it is to changes in the oxalacetate level.

Further examination of this phenomenon was made with a more complete model of the Citric Acid Cycle which allowed the acetylCoA: free CoA ratio to float. The two dummy reactions simulating the supply of acetylCoA and removal of free CoA were replaced by the reaction:

1.0 Supplier + CoA = 1.0 Supplier + acetyl CoA / k ; ..... (1)

where k was given a value which would maintain the flux through this reaction at the normal steady state value when {CoA} was at the 'normal' level (0.066 mmol/kg ratio). Changes in {CoA} concentration will now increase or decrease the rate of formation of acetylCoA: in this sense the reaction behaves like a simple enzyme. This hypothetical representation of the supply of acetylCoA is independent of whether the 'supplier' is

TABLE IV.iii.1. Effects of varying the AcetylCoA concentration.

INTERMEDIATE	CONCENTRATIONS (mmol/kg mito.)		
Acetyl CoA	0.033	0.066	0.10
Citrate	0.2179×10^{-1}	0.2431×10^{-1}	0.2535×10^{-1}
cis-Aconitate	0.8361×10^{-3}	0.9329×10^{-3}	0.9738×10^{-3}
Isocitrate	0.1337×10^{-2}	0.1491×10^{-2}	0.1555×10^{-2}
2-Oxoglutarate	0.7907×10^{-5}	0.8822×10^{-5}	0.9198×10^{-5}
SuccinylCoA	0.2195×10^{-2}	0.2455×10^{-2}	0.2563×10^{-2}
Succinate	0.2774×10^{-1}	0.3152×10^{-1}	0.3312×10^{-1}
Fumarate	0.9655×10^{-1}	0.9541×10^{-1}	0.9492×10^{-1}
Malate	0.4895×10	0.4837	0.4813
Oxalacetate	0.6210×10^{-4}	0.6114×10^{-4}	0.6074×10^{-4}
Flux at Steady State	1.1969×10^{-3}	1.3350×10^{-3}	1.3920×10^{-3}

All other conditions apart from acetylCoA concentration are as per 'normal' (i.e. as per Table IV.i.1).

carbohydrate, fat or ketone, while permitting conclusions to be made concerning the effect of the input rate of acetylCoA on the Cycle.

The dummy reaction used in the previous studies (section i) maintained the free concentration of CoA at 0.066 mmol/kg mito., regardless of the level at which (acetyl CoA) was set. Moreover, no account had been taken of enzyme-bound CoA, which appears as an 'invisible' source of CoA supplied by the dummy 'REM' enzyme. In order to make results exactly comparable with previous ones this was now allowed for by starting the simulation with an extra quantity of free CoA. A value of 0.0968 mmol/kg mito. was used. This distributed itself amongst the enzymes binding CoA in such a way that free CoA settled to a level of 0.066 mmol/kg mito. at the steady state.

In subsequent simulations the rate of flux through the acetylCoA 'supplier' reaction was changed to 50% and 200% of the normal steady state value by altering k in equation (1). The Sensitivities of the Cycle enzymes in all three situations were measured and are shown on Table IV.iii.2. In the simulations now being discussed, the Sensitivity of the 'supplier' enzyme could be measured in addition to the Sensitivities of the Citric Acid Cycle enzymes. The results indicated that the supply of acetylCoA can also be an important controller of the Cycle flux. In conditions comparable to the original simulation discussed in Section i. (designated 'normal'), the control of Cycle operation resided with the acetylCoA supplier and with citrate synthase in almost equal proportions. However when the rate of supply of acetylCoA was doubled, the majority of control lay with the citrate synthase reaction.

The fact that the Sensitivity value for citrate synthase under these conditions is very high (0.72) by definition means that the amount of citrate synthase is the important factor. In other words, citrate synthase has only a limited capacity to remove the increased acetylCoA by reacting it with oxalacetate to form citrate (and by implication, to increase the Cycle flux, since none of the other Cycle enzymes display

TABLE IV.iii.2. 'Sensitivities' of the Citric Acid Cycle Enzymes.

Floating AcetylCoA:free CoA ratio by using the reaction:-

$$CoA + Supplier \xrightarrow{k} AcCoA + Supplier$$

	Rate of Acetyl CoA Supplier Reaction		
	50%	NORMAL	200%
Acetyl CoA Supplier	0.74	0.44	0.11
Citrate Synthase	0.04	0.37	0.72
Aconitase	0	0	0
Isocitrate Dehydrogenase	0.01	0.03	0.05
2-Oxoglutarate Dehydrogenase	0	0	0
Succinyl Thiokinase	-0.04	-0.02	0
Succinic Dehydrogenase	0.006	0.03	0.05
Fumarase	0.002	-0.01	-0.01
Malate Dehydrogenase	0.04	0.02	0.02
Cycle Flux (% of 'normal')	69	100	117
AcetylCoA:free CoA	0.15	0.5	1.6

'Normal' flux through cycle is 1.2 μmoles/sec/kg mito.

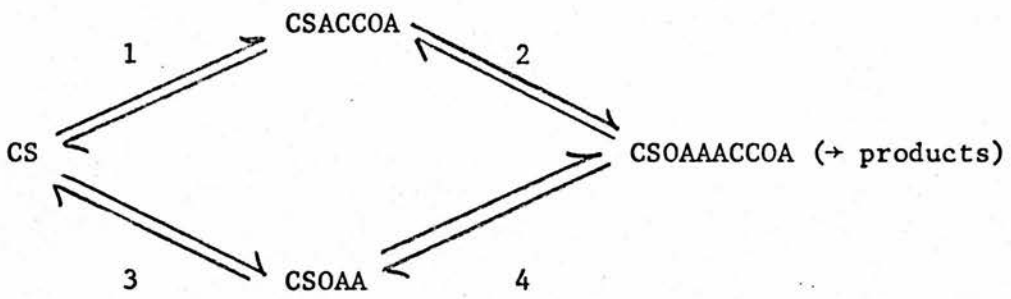
appreciable Sensitivities under these conditions). This is emphasised by the fact that doubling the rate of acetylCoA input only produces a 17% increase in Cycle flux.

The interesting point here is why this happens; why can the citrate synthase not support a much larger flux when {acetylCoA} is increased? The answer to this lies in the fact that the binding constants (K_m values) for both oxalacetate and acetylCoA towards citrate synthase are very small (5μM), thus both substrates bind very strongly to the enzyme. An examination of the output from the 'normal' simulation in Appendix III shows that the majority of citrate synthase is in the enzyme-acetylCoA complex (CSACCOA) form, whose concentration is almost an order of magnitude more than that of the free enzyme form (CS). Although oxalacetate also binds as strongly, its steady-state concentration is very low (ca. 6.2×10^{-5}mM) and hence the concentration of enzyme-oxalacetate form (CSOAA) is also very low. Correspondingly the amount of oxalacetate bound either to the free enzyme or to the enzyme-acetylCoA form to produce the ternary reactive complex (CSOAAACCOA) is also necessarily low.

Fig. IV.iii.1 shows a simplified diagram of the mechanism adapted for citrate synthase in the simulation studies. Calculation of the fluxes through each 'branch' of the mechanism which leads directly to the ternary reactive complex (CSOAAACCOA) (reactions 2 and 4 in Fig. IV.iii.1), using the values of the reacting species at steady state (Appendix III), shows that the 'branch' {enzyme → enzyme-acetylCoA → enzyme$<^{\text{acetylCoA}}_{\text{oxalacetate}}$} has much the highest flux. This then, is the major route of production of the ternary complex.

When the concentration of acetylCoA is increased, as in this study, the amount of enzyme-acetylCoA complex will obviously increase. However this increase cannot be as large as the increase in {acetylCoA}, since there is not enough free enzyme left to bind it all.

FIG.IV.iii.1. Simplified diagram of the Mechanism of Citrate Synthase.



Concentrations at Steady State (mmol/kg mito.).

CS	2.4287×10^{-3}
CSOAA	4.5028×10^{-6}
CSACCOA	1.7572×10^{-2}
CSOAAACCOA	4.5080×10^{-6}

Flux through Reaction 2. 1.0687×10^{-3}

Flux through Reaction 4. 1.2830×10^{-4}

All values taken from Appendix III.

That there is an increase in flux through citrate synthase at all (17%) is due predominantly to the increase in concentration of the enzyme - acetylCoA complex. In the Sensitivity study, the increase in flux produced when citrate synthase concentration was raised by 1% was a direct result of the increased capacity to form enzyme - acetylCoA complex. In simple terms the enzyme is almost saturated with acetylCoA.

One further point to be made here is the dependence of the major route of production of the ternary complex (reaction 2 in Fig. IV.iii.1) on oxalacetate concentration. Although in this study it was of no consequence since {oxalacetate} was not allowed to vary (NAD/NADH kept constant), it is in general an important parameter governing the flux through citrate synthase, especially in view of the fact that the enzyme was nearly saturated with acetylCoA in most of the simulation studies reported in this Chapter.

To return to Table IV.iii.2, when the rate of acetylCoA supply was below 'normal' the most Sensitive enzyme was the acetylCoA supplier. In these circumstances the Cycle controls its own operation to a very small extent; the flux is heavily dependent on the activity of the acetylCoA supplier as Citrate Synthase is not saturated with acetylCoA in this situation.

To some extent the conclusions drawn from this study might seem a statement of the obvious, i.e. when the rate of production of acetylCoA is low it is fed into the Cycle unhindered by citrate synthase, and the control of the Cycle flux lies primarily with the acetylCoA-producing enzyme. However, the different roles played by citrate synthase under the varying conditions indicate that it functions as a 'buffer' at the entry port to the Cycle with the effect that, where no other alterations in conditions arise, the flux through the Cycle cannot respond linearly to increases in acetylCoA concentration.

It should also be re-emphasised at this point that this model supplier does not represent any particular pathway of production of acetylCoA (i.e. from fat, carbohydrate or ketone precursors), and these conclusions therefore refer to acetylCoA supply in general, irrespective of the nature of the source. It should also be pointed out that {total-CoA} was set at an experimentally-determined level (Williamson & Corkey, 1969), and therefore the computed values of {acetylCoA} were within the physiological range. The computed {oxalacetate} was also within the experimental range.

Section iv. Cycle Control and the Ratio of the Pyridine Nucleotides

The preliminary experiments on the simulation of the Cycle discussed in Section i. suggested that the NAD^+:NADH ratio is very important in Cycle regulation. It was consequently essential that the effects of a 'floating' NAD^+:NADH ratio should be studied. To do this the simulation was used as in Section i. but the reaction holding the NAD^+:NADH ratio constant was replaced by a reaction simulating the oxidation of NADH by the Electron Transport Chain, thus:

$$NADH + 1.0\ ETS = NAD^+ + 1.0\ ETS\ /\ \underline{k} \qquad \ldots\ldots (2)$$

where, as in the study described in Section iii., $\underline{k}$ was set such that the flux through this reaction corresponds to the 'normal' steady-state value at the 'normal' concentration of NADH. The same principles apply to the use of this reaction as those discussed in the previous Section with reference to the acetylCoA supplier reaction. The Sensitivities of the Cycle enzymes and the 'ETS' enzyme (which represents the combined effect of the Electron Transport Chain components on the Cycle operation) were measured in the 'normal' situation and with the rate constant (k) for reaction (2) at 50% and 200% of the 'normal' value. The {acetylCoA}: {free:CoA} ratio was fixed by means of the two equations used in the study reported in Section i; it was <u>not</u> allowed to float.

The results are shown in Table IV.iv.1. In all three situations the most Sensitive enzyme is the 'ETS', with citrate synthase exhibiting a much reduced Sensitivity compared with previous studies, although it is still the most Sensitive enzyme of the Cycle proper. As might be expected from the studies reported in Section i, an increase in the NAD^+:NADH ratio elicits a larger increase in the Cycle flux than does an increase in the acetylCoA:freeCoA ratio. Doubling the flux through reaction (2) caused an increase in Cycle flux of 56%, whereas doubling the rate of reaction (1) brought about only a 17% increase in Cycle flux (Section iii). This study thus reemphasises the importance of the NAD^+:NADH ratio (and hence the concentration of oxalacetate) to the regulation of Cycle flux. As discussed in Section iii, citrate synthase is almost saturated with acetylCoA under 'normal' conditions, and the flux through the enzyme is very responsive to changes in the oxalacetate concentration.

One other point to note from this study is the appearance of Negative Sensitivities for some of the enzymes, especially isocitrate and malate dehydrogenases. This means that as the concentration of the enzyme was increased the flux through the Cycle decreased. This phenomenon could be explained by an examination of the NADH levels. Since the total amount of pyridine nucleotides is fixed (as it is in mitochondria), and as in the study discussed in this Section it is the <u>rate constant</u> for the conversion of NADH to NAD^+ which is set at the beginning of the simulation (not the ratio of NAD^+:NADH as in previous experiments), any increase in the amount of bound pyridine nucleotides as a result of increasing the concentration of a dehydrogenase automatically decreases the concentration of the unbound species in the system. Since NADH binds more strongly than NAD^+ to all of the NAD^+-linked dehydrogenases of the Citric Acid Cycle, increasing the enzyme concentrations by 1%

TABLE IV.iv.1. 'Sensitivities' of the Citric Acid Cycle Enzymes.

Floating the NAD^+:NADH ratio by using the reaction

$$NADH + ETS \xrightarrow{k} NAD + ETS$$

	Conversion Rate (k)		
	50%	'NORMAL'	200%
Citrate Synthase	0.213	0.276	0.316
Aconitase	-0.0014	0	0
Isocitrate Dehydrogenase	-0.131	-0.109	-0.08
2-Oxoglutarate Dehydrogenase	0	0	0
Succinyl Thiokinase	0	0	0.005
Succinic Dehydrogenase	0.007	0.017	0.037
Fumarase	-0.003	-0.008	-0.005
Malate Dehydrogenase	-0.128	-0.117	-0.096
'ETS'	0.743	0.677	0.610
Cycle Flux (% of 'normal')	61	100	156
NAD^+:NADH	0.528	0.887	1.447

'Normal' flux through cycle is 1.2 μmoles/sec/kg mito.

significantly increases the quantity of bound NADH but not bound NAD^+. When the new steady state is established a decrease in oxalacetate concentration following the increased sequestration of NADH causes the flux through the Cycle to decrease. The fall in NAD:NADH ratio which accompanies negative Sensitivity is paradoxical; it has been more fully investigated by Ottaway (1976). This is an interesting example of the 'Sols-Marco' effect (Sols & Marco, 1970) in which the NAD^+:NADH ratio is altered by increased sequestration, but in an opposite direction to that expected by the binding constants (cf. also Atkinson et al., 1975).

Section v. Cycle Control and the Rates of AcetylCoA supply and of NADH Reoxidation.

The natural progression from the studies so far discussed, was to run a simulation with both the NAD^+:NADH and acetylCoA:free CoA ratios allowed to 'float'. The enzyme Sensitivities were measured in three conditions: at the normal state, with the rate of acetylCoA production doubled and with the rate of NADH oxidation doubled. The results are shown in Table IV.v.1. Under the normal conditions, the majority of the Sensitivity lay with the 'ETS' enzyme, citrate synthase and the acetylCoA supplier both having a smaller but significant share of the control. Isocitrate and malate dehydrogenases have small but significant negative Sensitivities. When the NADH oxidation rate was increased (column 2 of the results) there was a marked increase in flux. The most Sensitive enzyme in this situation was the acetylCoA supplier, that is, the flux through the Cycle becomes much more dependent on the rate of supply of acetylCoA. This was a result of the increased NAD^+:NADH ratio which provided a higher steady-state concentration of oxalacetate, and thus there was less dependence on this intermediate (a fact supported by the decreased Sensitivity of 'ETS') and more dependence on acetylCoA

TABLE IV.v.1. 'Sensitivities' of the Citric Acid Cycle Enzymes.

Floating AcetylCoA:free CoA and NAD^+:NADH ratios

CoA → AcCoA ..... (1)

NADH → NAD ..... (2)

	CONVERSION RATES		
	(1) + (2) 'NORMAL'	(1) NORMAL (2) 200%	(1) 200% (2) NORMAL
Citrate Synthase	0.192	0.025	0.246
AcCoA Supplier	0.159	0.573	0.032
'ETS'	0.56	0.239	0.666
Isocitrate Dehydrogenase	-0.092	-0.025	-0.111
Malate Dehydrogenase	-0.100	-0.038	-0.111
2-Oxoglutarate Dehydrogenase	0	-	-
Succinic Dehydrogenase	0.008	-	-
Succinyl Thiokinase	-0.008	-	-
Cycle Flux	100%	133%	105%
% of 'normal'			
NAD^+:NADH	0.887	1.829	0.785
AcetylCoA:free CoA	0.5	0.126	1.88

'Normal' flux through cycle is 1.2 μmoles/sec/kg mito.

concentration for the turnover through citrate synthase and hence the flux through the Cycle. It should be noted, however, that the 'ETS' Sensitivity although diminished was still significant. Citrate synthase displayed a very small Sensitivity under these conditions and very little control was exercised within the Cycle proper; the Cycle was primarily controlled by factors external to it.

In the third situation, in which the input rate of acetylCoA was doubled, the most Sensitive enzymes were 'ETS' and citrate synthase. Here both the acetylCoA and oxalacetate concentrations were important factors in the flux through citrate synthase (and hence to Cycle flux). At the increased concentration of acetylCoA, citrate synthase was saturated with this substrate, which led to the high Sensitivity value for this enzyme. Under such conditions the flux through it became dependent on oxalacetate concentration (as discussed in Section iii). Since this is dependent on the ratio of NAD^+:NADH, the 'ETS' was the major site of control of flux through the Cycle.

There are important conclusions to be drawn from these results. In the simulations with 'fixed' coenzyme ratios (Section i.) citrate synthase was the only enzyme important for the regulation of flux, but from this last study it has become apparent that the rate of production of acetylCoA and the rate of reoxidation of NADH are also important to the control of the flux through the Cycle. Although under certain conditions these two factors can almost entirely abolish control by the enzymes within the Cycle proper, their effects are exerted through the concentrations of the substrates of the citrate synthase reaction i.e. acetylCoA and oxalacetate. All three factors, namely activity of citrate synthase, rate of acetylCoA production and the rate of NADH reoxidation can be important to the control of Cycle flux; the particular conditions under which the Cycle operates at any moment

determines which factor is the major site of Cycle regulation. If, say, one considered the situation where pyruvate dehydrogenase or β-oxidation were to proceed unhindered, the major control points of the Cycle flux would be both the 'ETS' which ultimately determines the oxalacetate concentration, and citrate synthase which would rapidly become saturated with acetylCoA.

One further point of general application should be emphasized with reference to this study. By simulating the system in greater detail (i.e. by encompassing more of the total mitochondrial system in the study), the numerical values of the Sensitivities of individual enzymes must fall because there are more control points, yet the sum total of Sensitivities controlling the system remains essentially the same. Since the control has to be distributed throughout more components of the system the numerical values of 'control strength' or 'Sensitivity' for each unit must become smaller. This does not imply that control points whose numerical ratings were measured in a more restricted system have become less important, merely that control must now be viewed in a wider perspective. For example, the reactions representing acetylCoA input and NADH reoxidation (Equations (1) and (2)) are themselves subject to further extraneous controls and should these systems be more fully simulated, control would be 'spread out' even more thinly throughout the system, yet the basic control of these parameters over the flux of the Citric Acid Cycle would be no less important. This difficulty inherent in the extension of the system under study, and the shortcomings of the definitions of control parameters has previously been pointed out by Kacser & Burns (1973) and by Ottaway (1974; 1976).

Section vi. Control of Ketone Body Metabolism in Heart Mitochondria

The pathway of breakdown of acetoacetate to two acetylCoA units involves the two enzymes 3-ketoCoA transferase (EC.2.8.3.5) and acetylCoA acetyltransferase also referred to as acetoacetylCoA thiolase (EC.2.3.1.9). Simulation models of these enzymes were constructed as detailed in Chapter II, and they were inserted into the Citric Acid Cycle model in place of the hypothetical acetylCoA supplier. The Cycle was then supplied with acetoacetate as a concentration of 1.0 mmol/kg mito. by means of a dummy reaction:

1.0 INPUT = 1.0 INPUT + 1.0 AcAc / 100.0 ..... (3)

When this system was simulated, the Cycle came to a halt after a few time units with almost all the carbon piled up in malate. Examination of the results led to the conclusion that this was happening because of a very low flux through 3-ketoCoA transferase. Almost all the succinylCoA produced by 2-oxoglutarate dehydrogenase was being transformed to succinate by succinyl thiokinase, and the fraction being used for activation of acetoacetate to acetoacetylCoA by CoA transferase was very low. As the Cycle slowed down because of the reduced acetylCoA input, the absolute amount of succinylCoA was decreased, which caused a progressive deterioration. Clearly in this simulation the thiokinase was too active for a favourable partition of succinylCoA between it and the ketone activation pathway. No metabolic effectors of succinyl thiokinase have been reported in the literature, apart from its own substrates and products, but this has never been a subject of intensive investigation. Also, information of the concentration of the enzyme within mitochondria and the V_{max} of the enzyme is not available. Although it is possible that such unknown factors might avert the difficulties uncovered by this simulation, it would be pointless or in fact impossible to investigate them in the simulation model without first

acquiring more information from experimental studies. What can be investigated and may equally well be a major cause of the problem, is the ratio of GTP/GDP.P_i. In all previous simulations this ratio was set at the same value as the ATP/ADP.P_i ratio through the reactions:

ATP = ADP + P / 470 / 10 ..... (4)

ATP + GDP = ADP + GTP / 100 / 100 ..... (5)

Reaction (5) represents the activity of Nucleoside Diphosphate Kinase which had been assumed to be at equilibrium. The value of the ATP/ADP.P_i ratio reported by Chance & Williams (1956) for State 3 conditions was used. This ratio implies a high concentration of ADP and a low concentration of ATP and consequently high GDP and low GTP concentrations. This makes the forward reaction of succinyl thiokinase very favourable indeed, and this was certainly one reason why there was a very small flux through CoA transferase when the acetoacetate pathway was inserted into the simulation model. Also, since the $K_m^{succinylCoA}$ value for succinyl thiokinase is 0.03mM (Cha & Parks, 1964) and for CoA transferase is 4.2mM (Hersh & Jencks, 1967) the steady state concentration of succinylCoA would have to be increased substantially before the CoA Transferase reaction could proceed effectively. By increasing the GTP/GDP.P_i ratio, the steady state distribution of the substrates and products of succinyl thiokinase could be altered, and the steady-state level of succinylCoA would then rise, allowing it to drive the CoA-transferase reaction more rapidly.

One way in which the GTP/GDP.P_i ratio could be altered is by changing the P_i concentration. However, in mitochondria, P_i not only participates in oxidative and substrate level phosphorylation, it also has an important function in membrane transport of metabolites (the 'cascade' system of membrane transport, see Chapter I, section iii), and it easily traverses the membrane via the Phosphate/Hydroxyl antiporter. It is a priori

unlikely that the concentration of P_i within the mitochondria alters greatly, or that it does in fact cause a significant change in the GTP/GDP.P_i ratio (and as a consequence, in the activity of succinyl thiokinase). For this reason it was decided that for the following simulation studies, where the ratios of GTP/GDP.P_i and ATP/ADP.P_i were to be altered, the concentration of P_i was to be held constant at the steady-state value used in previous studies (Sections i-iv), and only the concentrations of GTP, GDP, ATP, or ADP were to be altered in order to vary the phosphorylation ratios of the adenine and guanine nucleotides. All further references to these ratios will therefore pertain to the actual ratio of ATP:ADP and GTP:GDP with the P_i held at a concentration of 0.7 mmol/kg mito.

There are two ways on which the GTP:GDP ratio can be considered to change physiologically. Either this ratio is increased, while the ATP:ADP ratio remains at a low value, which would represent a situation where the nucleoside diphosphate kinase reaction (equation (5)) was not at equilibrium, or the ratios both of GTP:GDP and of ATP:ADP are increased, representing the situation in which nucleoside diphosphate kinase is at equilibrium, but where, even in State 3 mitochondria, the effective ATP:ADP ratio does not fall to the low values previously used in simulation.

A short preliminary study was carried out to find the order of magnitude of the ratio of GTP:GDP at which the input of acetoacetate could wholly support the Cycle operation. This was found to lie between 1.0 and 2.0. Consequently, when investigating the effects of changing the ratios of GTP:GDP and ATP:ADP, the value of 2.0 was used as a 'high' value.

To obtain a complete picture of how the acetoacetate input pathway affects the Cycle operation, the simulations with high GTP:GDP and high or low ATP:ADP ratios just outlined were repeated with the 'floating' acetylCoA supplier replacing the acetoacetate utilisation pathway. Thus, the study consisted of four parts as detailed in Table IV.vi.A. The results are necessarily very complicated and require discussion of a number of points, hence each part is discussed separately with respect to results of the previous Section (v.) and to the other parts of this Section.

Part 1. Input of AcetylCoA by Supplier Reaction with low ATP:ADP Ratio and high GTP:GDP Ratio.

The results of this study are shown in Table IV.vi.1 together with the same values for the simulation in Section v. under 'normal' conditions. The differences caused by having a raised GTP:GDP ratio are minor. All the effects can be attributed to the rise in the steady-state level of succinylCoA, which causes a decrease in the concentration of free CoA. The flux through the Supplier reaction is decreased, the ratio of acetylCoA:free CoA falls, and hence Cycle flux decreases.

Such effects are of little consequence. The major conclusions made in the previous Sections are not altered by elevating the GTP:GDP ratio. The 'sequestering' of free CoA as succinylCoA does not have any great effect on the Cycle when the GTP:GDP ratio is raised, so long as acetylCoA is produced by the dummy supplier reaction.

Part 2. Input of AcetylCoA by Supplier Reaction; Ratios of ATP:ADP and GTP:GDP both High.

This study investigated the situation in which the nucleoside diphosphate kinase reaction was assumed to be in equilibrium. The results are shown in Table IV.vi.2. Any differences between this and the last study must be due to the increased level of ATP, since ADP was not

TABLE IV.vi.A. Outline of the Studies in Section vi.

PART/TABLE	SOURCE of AcetylCoA	GTP:GDP Ratio	ATP:ADP Ratio
1	Dummy Supplier	High	Low
2	" "	High	High
3	Acetoacetate Pathway	High	Low
4	" "	High	High

TABLE IV.vi.1. AcetylCoA Supplied by Dummy Reaction, ATP:ADP Ratio Low, GTP:GDP Ratio High.

Parameter	'NORMAL' (values from Section v.)	This Study
CONDITIONS AT STEADY STATE		
AcetylCoA:freeCoA	0.5	0.412
ATP:ADP	0.015	0.02
GTP:GDP	0.015	2.00
NAD^+:NADH	0.887	0.927
Cycle Flux (% of 'normal')	100%	98.5%
ENZYME SENSITIVITIES		
Citrate Synthase	0.192	0.170
Isocitrate Dehydrogenase	-0.092	-0.076
2-Oxoglutarate Dehydrogenase	0.0	0.0
Succinyl Thiokinase	-0.008	0.0
Succinic Dehydrogenase	0.008	0.017
Malate Dehydrogenase	-0.10	-0.085
AcetylCoA Supplier	0.159	0.204
'ETS'	0.56	0.509
INTERMEDIATES CONCENTRATIONS		
SuccinylCoA	2.1957×10^{-3}	8.0250×10^{-3}
Oxalacetate	6.2098×10^{-5}	6.4466×10^{-5}
AcetylCoA	3.2999×10^{-2}	2.6788×10^{-2}
free CoA	6.6001×10^{-2}	6.5005×10^{-2}

TABLE IV.vi.2. AcetylCoA Supplied by Dummy Reaction. ATP:ADP and GTP:GDP Ratios Both High.

CONDITIONS AT STEADY STATE	
AcetylCoA:free CoA	0.379
ATP:ADP	2.0
GTP:GDP	2.0
NAD^+:NADH	1.145
Cycle flux (% of normal)	100.8%
SENSITIVITY OF ENZYMES	
Citrate Synthase	0.150
Isocitrate Dehydrogenase	0.033
2-Oxoglutarate Dehydrogenase	0.008
Succinyl Thiokinase	0.008
Succinic Dehydrogenase	0.025
Malate Dehydrogenase	-0.075
AcetylCoA Supplier	0.249
'ETS'	0.464
INTERMEDIATES CONCENTRATIONS (mmol/kg mito.)	
SuccinylCoA	8.4022×10^{-3}
Oxalacetate	7.1572×10^{-5}
AcetylCoA	2.5220×10^{-2}
free CoA	6.6542×10^{-2}

simulated as interacting with any of the Cycle enzymes. ATP, on the other hand, was included as an inhibitor of isocitrate dehydrogenase, the inhibition being competitive with respect to both NADH and NAD^+ for the free enzyme.

When we compare the results with the previous study (Part 1), we find an increase in the NAD^+:NADH ratio, a decrease in the acetylCoA: free CoA ratio and a slight increase in flux even from the normal (Section iv.) situation. These facts are explicable as consequences of the enhanced binding of ATP to isocitrate dehydrogenase. This displaces bound NADH and NAD^+, and consequently the free concentration of these increases. By the same principle which was outlined in Section iv., but this time in reverse, the ratio of NAD^+:NADH increases. This results in an increased steady-state concentration of oxalacetate, which brings about a rather paradoxical increase in Cycle flux when the ATP concentration is raised 100-fold.

As expected from the studies in Section v. (Table IV.v.1, column 2) an increase in the NAD^+:NADH ratio also results in lower Sensitivities for 'ETS' and citrate synthase, and a raised Sensitivity for the acetylCoA Supplier, although these effects are slight. One Sensitivity which is noticeably different from the normal (see Table IV.vi.1, column 1) is that for isocitrate dehydrogenase. This enzyme now shows a small, but positive Sensitivity. Again this effect is due to the high ATP concentration which reduces the effective concentration of the enzyme. This leads to the Cycle flux becoming to some extent dependent on the flux through isocitrate dehydrogenase, but even at this elevated ATP concentration, isocitrate dehydrogenase can still by no means be considered a major control point of Cycle Flux.

Neither of the studies in Parts 1 or 2 show any major difference from previous studies, indicating that the ratios of ATP:ADP and GTP:GDP

have very minor effects on the Cycle as it has been simulated up to this point, i.e. with 'acetyl-CoA Supplier' as the source of the carbon atoms oxidized to CO_2.

Part 3. Effects on the Citric Acid Cycle of Acetoacetate Input with High GTP:GDP and Low ATP:ADP Ratios.

In this study the simulation contained the pathway for input of acetoacetate, together with a high ratio of GTP:GDP and a low ratio of ATP:ADP. The results are shown in Table IV.vi.3.

The ratio of acetylCoA:free CoA dropped markedly to 0.18, much lower than the normal value of 0.5. This was due to a large drop in the concentration of acetylCoA and of its rate of production through the transferase and thiolase enzymes. The latter was not brought about by a decrease in free CoA concentration, but by the dependence of the transferase reaction on the availability of succinylCoA. In Part 1 of this Section on the other hand, the slightly slower than normal rate of acetylCoA production by the Supplier found when the GTP:GDP ratio was high, was caused by a decrease in free CoA concentration. A change from dependence on free CoA to dependence on succinylCoA is supported by the Sensitivity values for the transferase and Thiolase enzymes: the transferase showed a very high Sensitivity under these conditions, but the Thiolase none at all. The large negative Sensitivity of succinyl thiokinase is also an effect of the dependence of the transferase reaction on the availability of succinylCoA. These Sensitivity values can be explained by considering the relative sequestration of succinylCoA by succinyl thiokinase and 3 ketoacylCoA Transferase. When the concentration of Succinyl Thiokinase is increased by 1% it binds more succinylCoA, so that the free succinylCoA concentration decreases slightly. This in turn means there is less succinylCoA for the transferase reaction and hence the rate of production of acetylCoA is

TABLE IV.vi.3. AcetylCoA produced from Acetoacetate. ATP:ADP Ratio Low, GTP:GDP Ratio High.

STEADY STATE CONDITIONS	
AcetylCoA:free CoA	0.178
ATP:ADP	0.02
GTP:GDP	2.00
NAD^+:NADH	1.10
Cycle flux (% of normal)	90.8%

SENSITIVITIES OF ENZYMES	
Citrate Synthase	0.147
Isocitrate Dehydrogenase	-0.083
2-Oxoglutarate Dehydrogenase	0.0
Succinyl Thiokinase	-0.681
Succinic Dehydrogenase	-0.267
Malate Dehydrogenase	-0.110
CoA Transferase	0.820
AcetoacetylCoA Thiolase	0.0
'ETS'	0.653

INTERMEDIATE CONCENTRATIONS (mmol/kg mito.)	
SuccinylCoA	6.1466×10^{-3}
Oxalacetate	7.8158×10^{-5}
AcetylCoA	1.7195×10^{-2}
free CoA	9.6549×10^{-2}

slowed down, and the Cycle flux decreases. The opposite situation arises when the concentration of the transferase is increased by 1%; the production of acetylCoA is increased and the Cycle flux rises.

The larger than normal negative Sensitivity of succinic dehydrogenase can be explained along similar lines to the negative Sensitivity of the thiokinase and transferase. When the concentration of succinic dehydrogenase is increased by 1% the concentration of free succinate decreases. This causes a slight decrease in free succinylCoA concentration, due to a change in the steady-state distribution of the substrates and products of succinyl thiokinase.

It is noteworthy that the 'ETS' still has a high Sensitivity under these conditions indicating that oxalacetate concentration remains an important regulator of Cycle flux. This is supported also by the fact that citrate synthase retains a significant Sensitivity. Thus in this situation it can be seen that even when the rate of acetylCoA supply is limiting the Cycle is still dependent on other factors, principally the NAD^+:NADH ratio and hence the oxalacetate level, for its activity. It also demonstrates that the control of Cycle flux can be a complex process. This could happen in an <u>in vivo</u> situation, where for one reason or another unconnected with the GTP:GDP ratio, the input of acetylCoA is curtailed and perhaps the requirement for ATP is not so great, hence NADH is not being reoxidised very rapidly. The Cycle flux will slow down under the influence of both factors; it is not essential that either of them should be dominant, as their effects are additive. The fact that Succinyl thiokinase is so sensitive in this particular study is entirely due to the way in which acetylCoA is produced from ketone bodies. However the results have a more general significance, because they show how 'supply' and 'demand' controls are co-ordinated in their effect on the Cycle.

Although the differences between this study and previous studies (Part 1 of this Section and Sections iii & v) may emphasize that the hypothetical acetylCoA Supplier used previously did not truly simulate a 'real' acetylCoA Supplier system now used for the first time, it must be remembered that every route of acetylCoA supply (whether it be from β-oxidation, pyruvate dehydrogenase or from ketone body degradation) will be dependent on extraneous factors which were purposely not included in the hypothetical supplier (see Section iii). In the case under discussion the important factor is succinylCoA, but in other situations it would be quite different factors (e.g. the pyruvate dehydrogenase, the NAD^+:NADH ratio). One may recall, however, a remarkable similarity between this case and one in Section ii. (Table IV.iii.2, column 1) where there was an acetylCoA:freeCoA ratio of 0.15, and the Sensitivity value of the hypothetical Supplier was 0.74. This suggests that when the constraints are similar to those now being presented, at least, the hypothetical 'supplier' represents the control features of the pathways in vivo which produce acetylCoA fairly accurately, even though they are more complex than the simulations represented them to be. This good agreement in one instance gives one confidence that the results in other conditions may also reproduce the situation in vivo with some accuracy.

Part 4. Effects of the Citric Acid Cycle of Acetoacetate Input with High GTP:GDP and High ATP:ADP Ratios.

Results of this study are shown in Table IV.vi.4. Much of the discussion in Part 3 is also applicable to this study. There are slight differences between the previous study and this one, but they are entirely due to the inhibition of isocitrate dehydrogenase by ATP. This causes an increase in free NADH concentration, followed by an alteration in the NAD^+:NADH ratio, a slightly decreased Sensitivity for ETS, increased flux, and the appearance of a positive Sensitivity for isocitrate dehydrogenase.

TABLE IV.vi.4. AcetylCoA produced from Acetoacetate. ATP:ADP and GTP:GDP ratios high.

STEADY STATE CONDITIONS	
AcetylCoA:free CoA	0.178
ATP:ADP	2.0
GTP:GDP	2.0
NAD^+:NADH	1.33
Cycle flux (% of normal)	93.1%

SENSITIVITIES OF ENZYMES	
Citrate Synthase	0.135
Isocitrate Dehydrogenase	0.072
2-Oxoglutarate Dehydrogenase	0.0
Succinyl Thiokinase	-0.682
Succinic Dehydrogenase	-0.26
Malate Dehydrogenase	-0.099
CoA Transferase	0.861
AcetoacetylCoA Thiolase	-0.242
'ETS'	0.601

INTERMEDIATE CONCENTRATIONS (mmol/kg mito.)	
SuccinylCoA	6.3035×10^{-3}
Oxalacetate	8.6260×10^{-5}
AcetylCoA	1.7191×10^{-2}
free CoA	9.6582×10^{-2}

There is only one other difference between this study and the last, which might not be expected from comparing Parts 1 of this Section, with Part 2, namely, thiolase now has a negative Sensitivity. This is caused by an increase in the binding of acetylCoA to the enzyme which is added. At the lower acetylCoA concentration now existing, this becomes important, and it alters the throughput of the Cycle.

A point that should be stressed here which is also true for the previous study is that if the GTP:GDP ratio were to be increased or the P_i concentration to be decreased (i.e. the GTP/GDP.P_i ratio to be increased) from the values used here, the Sensitivities of the transferase and thiokinase enzymes and the acetylCoA:freeCoA ratio, would approach more 'normal' values. No exhaustive studies on this were undertaken, but Table IV.vi.5 gives the results of one study, which indicates what might happen. The GTP/GDP.P_i ratio was changed by decreasing the P_i concentration. The results led to the conclusion that, as with the previously used 'hypothetical' acetylCoA Supplier, the reaction producing acetylCoA is only very 'Sensitive' so long as the acetylCoA supply is the limiting factor in the Cycle operation. When the strain is removed from the supplier system (in this instance when the succinylCoA level is increased), it ceases to become a major regulation site for the flux through the Citric Acid Cycle. The same conclusion would clearly hold in principle for any other mechanism by which acetylCoA could be supplied to the Cycle.

TABLE IV.vi.5. Input of acetoacetate with a Low P_i Concentration, a Low Ratio of ATP:ADP and a High Ratio of GTP:GDP.

STEADY STATE CONDITIONS	
AcetylCoA:free CoA	2.25
ATP:ADP	0.02
GTP:GDP	2.00
P_i (conc.)	0.22 mmol/kg mito.
NAD^+:NADH	0.788
Cycle flux (% of normal)	106%

SENSITIVITIES OF ENZYMES	
Citrate Synthase	0.261
Isocitrate Dehydrogenase	-0.11
2-Oxoglutarate Dehydrogenase	0.0
Succinyl Thiokinase	-0.024
Succinic Dehydrogenase	0.0
Malate Dehydrogenase	-0.11
CoA Transferase	0.032
AcetoacetylCoA Thiolase	0.0
'ETS'	0.68

Section vii. Simulation of Nucleoside Diphosphate Kinase

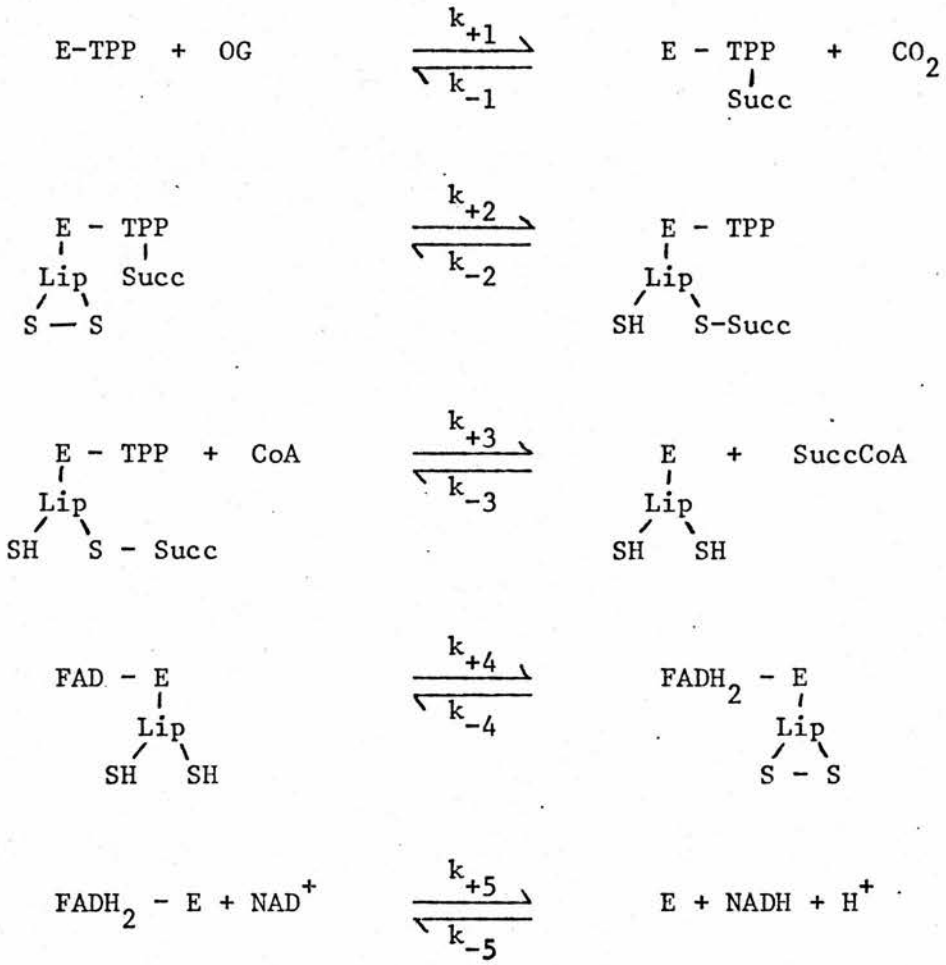
These studies reported in the last Section were enlightening with respect to the control of the Cycle when the ratios of ATP:ADP and GTP:GDP are elevated, but the fundamental problems a) what these ratios are in the mitochondrion and how they are related to one another; and b) what the steady state concentration of succinylCoA is during Cycle operation, still remain. These problems are particularly important when acetoacetate is the source of acetylCoA. The former point may be posed in terms of the ability of nucleoside diphosphate kinase to equilibrate the phosphorylation potential of the adenine and guanine nucleotides in mitochondria: the latter point relates to the activity of succinyl thiokinase. Both basically lead to the question of the true value of the GTP/GDP.P_i ratio. To investigate this further a simulation of nucleoside diphosphate kinase was carried out as described in Chapter II. When this enzyme was simulated in isolation, using similar concentrations of ATP, ADP, GTP and GDP to those used in this Chapter, the enzyme did maintain the equilibrium between the ATP:ADP and GTP:GDP ratios predicted from the equilibrium constant (1.0) used. However, when the equation deck for this enzyme was inserted into the Citric Acid Cycle model (with acetylCoA supplied by the dummy reaction), it did not maintain equilibrium between its substrates and products at the steady state. Over a wide range of ATP:ADP ratios, from 0.02 to 2.0, the GTP:GDP ratio was always significantly higher than the ATP:ADP ratio, e.g. when the ATP:ADP ratio was 1.0, the GTP:GDP ratio was 3.365. This was because in isolation the enzyme has infinite time to reach equilibrium, but in the kinetic situation within the Citric Acid Cycle simulation it has to cope with a certain flux for which not enough enzyme molecules are available, and so equilibrium is never achieved. The Sensitivity of nucleoside disphosphate kinase under these conditions would clearly be high.

When the acetoacetate utilisation pathway was included, the nucleoside diphosphate kinase reaction approached its equilibrium much more closely. At an ATP:ADP ratio of 1.0, the GTP:GDP ratio fell below the critical value of 2.0, as a result of the fact that the flux through succinyl thiokinase was only about 50% of its usual value, so that the rate of production of GTP was halved. Nucleoside diphosphate kinase was under less pressure, and so phosphate exchange between ADP and GTP was nearer to the rate that the enzyme, at the concentration used, is capable of supporting.

The fact that nucleoside diphosphate kinase did not support equilibration between the adenine and guanine nucleotides in these simulations presents one problem of general importance. In all the simulation reported in this Chapter, the formation of ATP and ADP and P_i was represented by a dummy reaction (reaction (4)) which is also a sink for ATP. However, in the mitochondrion this reaction is proceeding unidirectionally, and probably at a rate much faster than the simulation allowed for. Thus in the *in vivo* situation nucleoside diphosphate kinase has to cope not only with the pressure of GTP production by succinyl thiokinase, but also the pressure of ATP production (and ADP removal) by oxidative phosphorylation. In this respect these studies with nucleoside diphosphate kinase included are unrealistic. To obtain a more accurate insight into the relationship between the ratios of the adenine and guanine nucleotides and their equilibration, or lack of equilibration, the electron transport chain and oxidative phosphorylation would have to be fully simulated.

While this would indeed be a useful study to carry out, it is not within the scope of this project, whose basic aims were to investigate the control of the Citric Acid Cycle. Moreover, there was not sufficient time for a study of this magnitude to be undertaken. Thus the question of the relationship between the phosphorylation ratios of the adenine and guanine nucleotides, like the question of the activity of succinyl thiokinase,

FIG.IV.viii.1. Reaction mechanism for 2-oxoglutarate dehydrogenase as proposed by Sanadi (1963).



Abbreviations: TPP, thiamine pyrophosphate; OG, 2-oxoglutarate; Succ, succinyl residue; Lip, Lipoate residue; SuccCoA, Succinyl CoA.

must remain unsolved until more detailed experimental and simulation investigations can be performed.

Section viii. Kinetic Studies on the 2-Oxoglutarate Dehydrogenase from Pig Heart

Introduction

While carrying out the survey of the papers in the literature relating to 2-oxoglutarate dehydrogenase, for the construction of a simulation model, it was found that the most complete kinetic analysis was reported as long ago as 1960 by Massey, who measured the K_m and V_{max} values for the enzymic reaction in the direction of NADH production. Other kinetic studies on the pig heart enzyme have been solely concerned with the measurement of K_m (or K_i) values for one or more substrates (or products) in the presence of saturating concentrations of the other substrates (Kanzaki et al., 1969; Johnson & Connelly, 1972; Smith et al., 1974). The values for the kinetic constants which they reported do not agree with each other or with Massey's results, but this may not be significant as the conditions of assay were different in each case.

A catalytic mechanism for this enzyme complex was proposed by Sanadi (1963) on the basis of investigations of the roles and locations of the cofactors, thiamine pyrophosphate (TPP) lipoic acid and FAD within the complex, and of the coenzymes NAD^+ and CoA which participate in the reaction (Sanadi, 1963). This mechanism is shown in Fig. IV.viii.1. In Cleland's (1963) terminology it can be described as a Hexi Uni Ping Pong mechanism, i.e., the first product (CO_2) is released before the second substrate (CoA) binds and the second product (succinylCoA) is released before the third substrate (NAD^+) binds.

A simulation using the Sanadi mechanism and Massey's kinetic parameters was set up as described in Chapter II, but in view of the apparent lack of control features exhibited by 2-oxoglutarate dehydrogenase in the

preliminary simulation of the Citric Acid Cycle, and the demonstration that NADH is an important factor in the control of the flux through the Cycle, it was felt desirable to check that the mechanism and the kinetic parameters actually represent the behaviour of the enzyme complex. The dehydrogenase needs to be simulated accurately if its importance as a Cycle regulation point is to be assessed.

The check was carried out by steady state kinetic measurements, using the system put forward by Fromm (1967) as described in Chapter III.

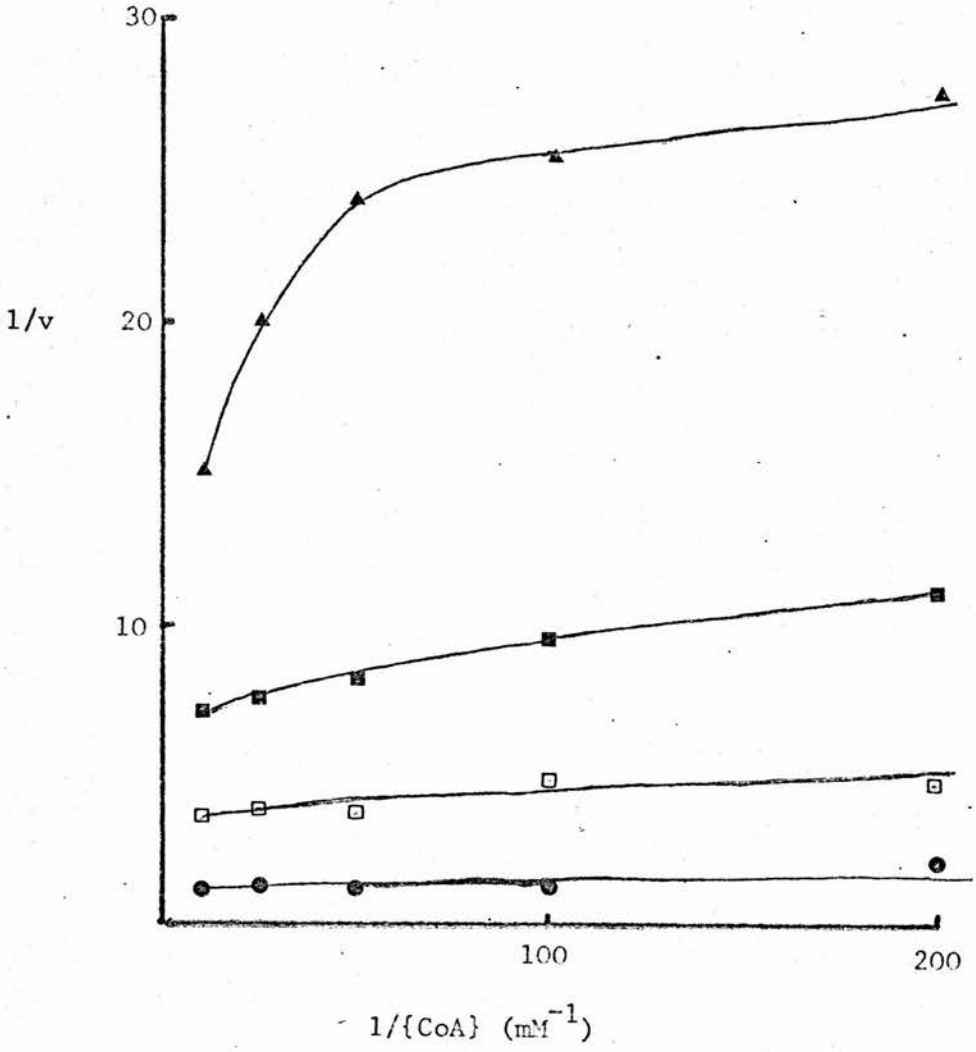
Results

Three sets of initial rate studies were performed. In all three cases, when CoA or NAD^+ was the variable substrate the double reciprocal plots showed considerable non-linearity (Figs. IV.viii.2 and 3). When 2-oxoglutarate was the variable substrate the reciprocal plots were linear. In the first two sets of assays the reciprocal plots with variable 2-oxoglutarate appeared to be parallel. However in the third set, in which the concentrations of NAD^+ were slightly higher than in the previous two sets, they were slightly converging (Figs. IV.viii.4 and 5).

According to Fromm's (1967) hypothesis, when a set of reciprocal plots is parallel, there is a release of product between the addition of the variable and the two fixed substrates (i.e. a Ping Pong mechanism is operating). Since the kientic studies on 2-oxoglutarate dehydrogenase yielded (almost) parallel linear plots with 2-oxoglutarate as the variable substrate, it was assumed that the first product (CO_2) leaves the enzyme surface prior to the binding of CoA and NAD^+.

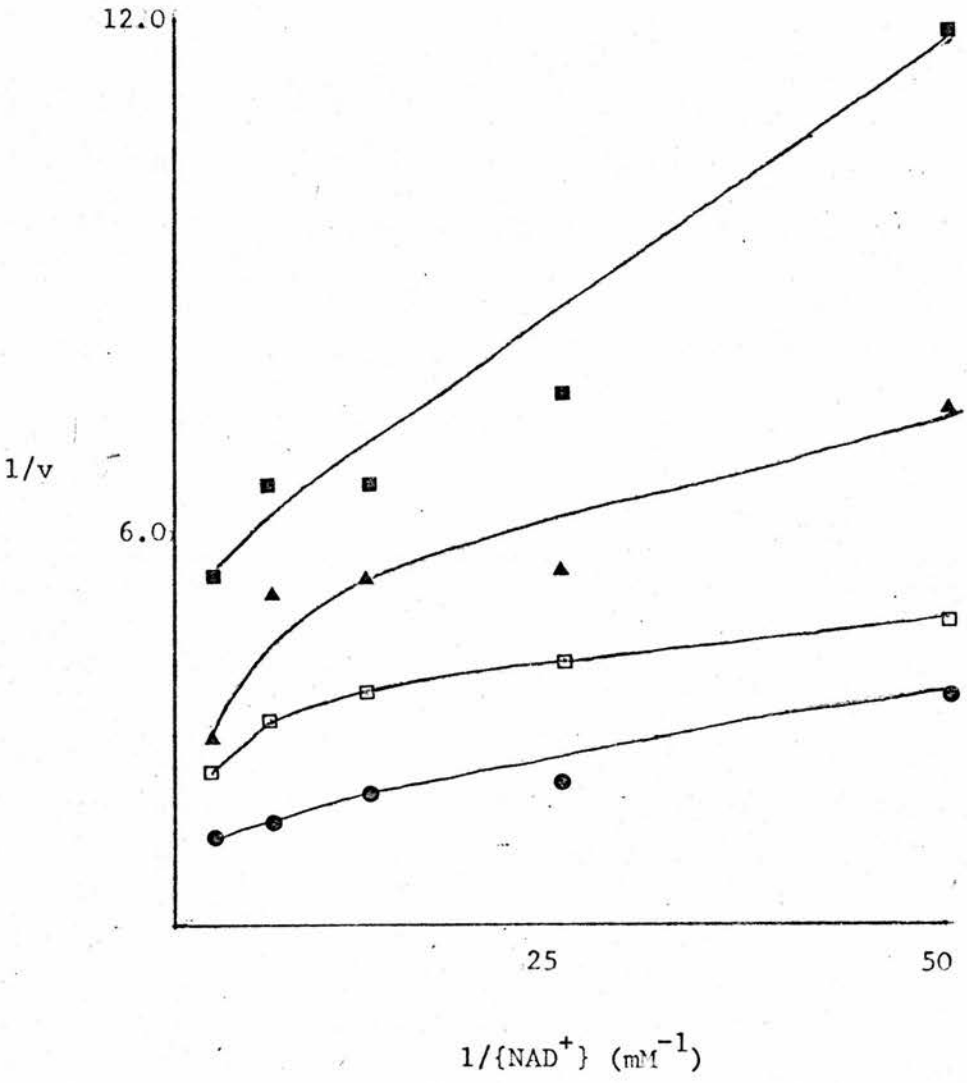
The curvatures of the reciprocal plots for CoA or NAD^+ as the variable substrate made it impossible to apply Fromm's (1967) rules to them.

FIG.IV.viii.2. Initial velocity study of 2-oxoglutarate dehydrogenase: Lineweaver-Burk plot of the data with CoA as the variable substrate.



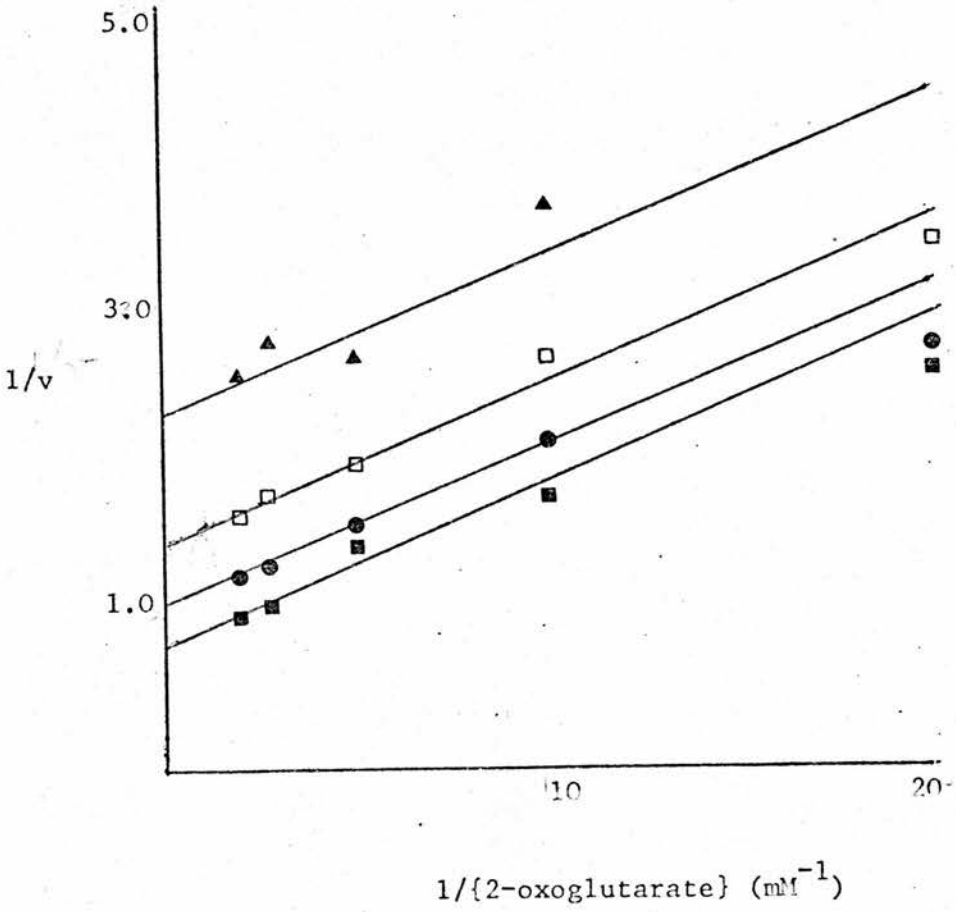
Points shown on the graph are the average of two measurements. Velocities expressed as μmoles NADH produced /min/mg protein. Concentrations of the 'fixed' substrates are 2-oxoglutarate, 0.5mM and NAD^+, 0.4mM (●); 2-oxoglutarate, 0.1mM and NAD^+ 0.08mM (□); 2-oxoglutarate, 0.05mM and NAD^+, 0.04mM (▣); 2-oxoglutarate, 0.025mM and NAD^+ 0.02mM (▲). Lines fitted by eye.

FIG.IV.viii.3. Initial velocity study of 2-oxoglutarate dehydrogenase: Lineweaver-Burk plot of data where NAD^+ is the variable substrate.



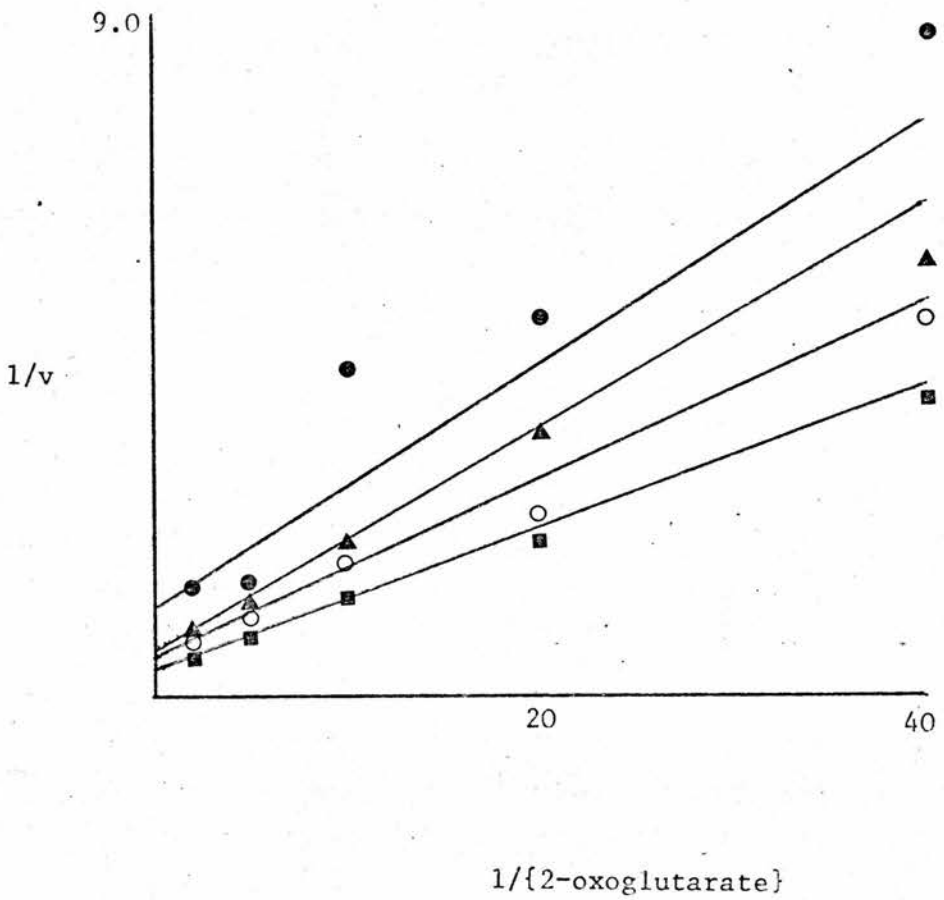
Points shown on the graphs are the average of two measurements. Velocities expressed as μmoles NADH produced /min/mg protein. Concentrations of the 'fixed' substrates are 2-oxoglutarate, 0.05mM and CoA, 0.05mM (●); 2-oxoglutarate, 0.2mM and CoA 0.02mM (□); 2-oxoglurarate, 0.1mM and CoA 0.01mM (▲); 2-oxoglutarate, 0.05mM and CoA 0.005mM (■). Lines fitted by eye.

FIG.IV.viii.4. Initial velocity study of 2-oxoglutarate dehydrogenase: Lineweaver-Burk plots of data where 2-oxoglutarate is the variable substrate (1).



Points shown on the graph are the average of two measurements. Velocities are expressed as μmoles NADH produced /min/mg protein. Concentrations of the fixed substrates are: NAD^+, 0.333mM and CoA 0.05mM (■); NAD^+, 0.133mM and CoA 0.02mM (●); NAD^+, 0.066mM and CoA 0.01mM (□); NAD^+, 0.033mM and CoA, 0.005mM (▲). Lines fitted using computer program as described in Chapter III.

FIG.IV.viii.5. Initial velocity study of 2-oxoglutarate dehydrogenase: Lineweaver-Burk plots of data where 2-oxoglutarate is the variable substrate (2).



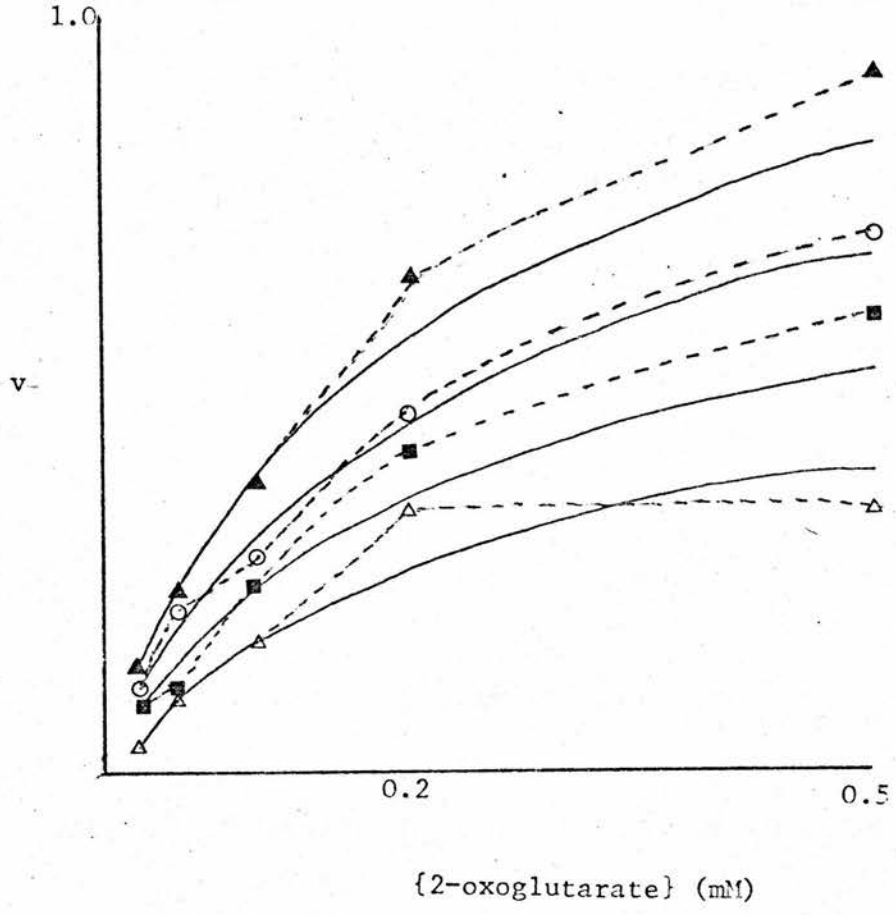
Points shown on the graph are the average of two measurements. Velocities are expressed as μmoles NADH produced /min/mg protein. Concentrations of the fixed substrates are: NAD^+, 0.4mM and CoA, 0.05mM (■); NAD^+, 0.2mM and CoA, 0.025mM (o); NAD^+, 0.08mM and CoA 0.01mM (▲); NAD^+, 0.04mM and CoA, 0.005mM (●). Lines fitted by computer program as described in Chapter III.

Analysis of Results

The analysis of steady-state kinetic measurements which do not yield linear reciprocal plots is rarely treated in any detail in publications dealing with kinetic analysis (e.g. Cleland, 1963; Fromm, 1967). Recently, Bardsley & Childs (1975) have suggested a method of analysing non-linear double reciprocal plots. This is essentially an analysis of $\underline{v}$ against $\underline{s}$ curves for points of inflexion and turning points, to gain information about the degree to which the substrate terms are raised in the numerator and denominator of the rate equation. This analysis was applied to the experimental results of the initial rate studies, but very variable patterns of $\underline{v}$ against $\underline{s}$ curves were produced (see Fig. IV.viii.6). Because of experimental error involved in the measurements of initial velocities, it is impossible to determine exactly which points the $\underline{v}$ against $\underline{s}$ curve should pass through. For this method to work, a large number of replicates of each measurement would have to be performed so that the value of the initial velocity at a particular substrate concentration could be obtained to a very high degree of accuracy. In this study only duplicate measurements were made, and even a slight discrepancy in one measurement could alter the mean value enough to form a point of inflection on the curve.

Analysis of results of the initial velocity studies by Bardsley & Childs' method becomes dependent on how the $\underline{v}$ against $\underline{s}$ curves are drawn. Fig. IV.viii.6 shows $\underline{v}$ against $\underline{s}$ plots for the initial rate studies with 2-oxoglutarate as the variable substrate for three of the sets of initial velocities; curves can be drawn to indicate multiple points of inflexion, yet all four sets of data nevertheless correspond very well to rectangular hyperbolas as shown. When this data was treated by Cleland's computer program (Gardiner & Ottaway (1969)), the data corresponded to rectangular hyperbolas within statistical significance levels (i.e., there was no deviation from linearity in the Wilkinson plots).

FIG.IV.viii.6. Initial rate studies of 2-oxoglutarate dehydrogenase: v against s plots where 2-oxoglutarate is the variable substrate.



Points indicated on the graph are the averages of two measurements. Velocities expressed as μmoles NADH produced /min/mg protein. Concentrations of fixed substrates are: NAD^+, 0.04mM and CoA, 0.005mM (Δ); NAD^+, 0.08mM and CoA, 0.01mM (■); NAD^+, 0.2mM and CoA, 0.025mM (o); NAD^+, 0.4 mM and CoA, 0.05mM (▲). Full lines represent those fitted according to the computer program for fitting reciprocal plots. Dotted lines drawn by eye.

The method of Bardsley & Childs thus could not be applied to the results of the initial velocity studies; very much more data would have been required (i.e. more data points per assay and more replicates of each point measured) for the method to be successful. Since there are three substrates involved in the enzyme reaction, acquiring sufficient data and indeed the number of replots and tests which would be required to analyse the results properly by Bardsley and Childs' method would be extremely time-consuming and tedious.

Since analysis of the steady-state data by graphical means, i.e., by Fromm's or Bardley & Childs' method could not yield enough information to formulate a mechanism for 2-oxoglutarate dehydrogenase, it was decided to use a different approach. This was to take the initial rate data and examine how well this is fitted by the rate equations corresponding to various possible mechanisms. There is a fairly new approach but one which is becoming increasingly necessary as more complex enzymic reactions are being studies (c.f. Reich, 1974; Feraudi et al., 1975, 1977; However, there is as yet no accepted method of performing such analyses and there are certain drawbacks in that a number of assumptions have to be made (e.g. that all substrates of the reaction are known). Moreover, the possible mechanisms have to be proposed before analysis, and this is subject to human bias in that plausible mechanisms can be overlooked. In spite of these drawbacks,it was decided to proceed with this type of analysis since no other method seemed likely to give further information about the mechanism of 2-oxoglutarate dehydrogenase from the available experimental data.

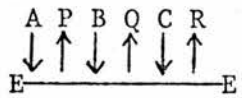
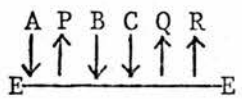
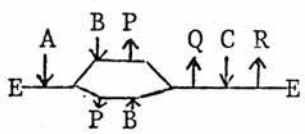
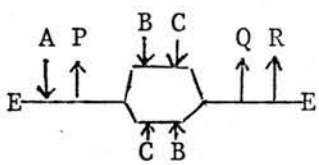
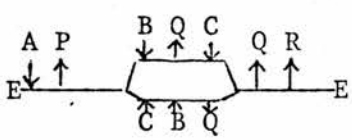
The method used was that of 'total fit', i.e., the fit of the complete set of initial velocity measurements to the rates predicted by (steady-state) initial velocity equations corresponding to several kinetic mechanisms was examined using the SIMPLEX optimization procedure as described in Chapter III.

Five mechanisms were chosen to be fitted to the data. These are shown in Fig. IV.viii.7. In the construction of these mechanisms certain assumptions were made, a) that 2-oxoglutarate binds before the other two substrates, b) that no dead-end complexes are formed, c) that the steady-state stoichiometry of the binding sites for the substrates is 1:1:1 and d) that 2-oxoglutarate cannot bind until NADH is released. Although it is obvious that succinylCoA cannot be formed until CoA has bound and that NADH cannot be formed until NAD^+ binds, these could also be considered as assumptions. Mechanism 1 is the Hexa Uni Ping Pong mechanism proposed originally by Sanadi. Although the Fromm analysis predicts that this is unlikely to be the mechanism of the enzyme, it was useful as a basic model with which other mechanisms could be compared. Also, as this is the mechanism that has previously been accepted for this enzyme complex, it was necessary to test any new proposed mechanisms for improvement over this one. There is no point in proposing a new mechanism for an enzyme if in fact it is no better a description of the enzymic reaction than existing mechanisms, particularly where previous suggestions have been drawn from wider-ranging studies with the enzyme.

Mechanism 2 has a sequential binding of CoA and NAD^+. From the information that can be gained from graphical analysis of the initial rate data, this is as plausible a mechanism as the Hexa Uni Ping Pong, and therefore must be included, although the Fromm analysis predicts that it is also unlikely.

The non-linearity of the double reciprocal plots with NAD^+ or CoA as the variable substrate suggests, in fact, that the binding of these substrates may be random, thus mechanisms 4 and 5 were constructed on these lines; mechanism 4 incorporates only a sequential binding of CoA and NAD^+, with either substrate binding first, mechanism 5 has both a sequential and a ping pong branch in the random binding sequence.

FIG.IV.viii.7. Mechanisms fitted to the initial rate data for 2-oxoglutarate dehydrogenase.

No.	Mechanism	Initial Rate Equation
1		$v = \frac{V_{max}.A.B.C}{K_m^c.A.B + K_m^b.A.C + K_m^a.B.C + A.B.C}$
2		$v = \frac{V_{max}.A.B.C}{K_m^c.K_i^b.A + K_m^c.A.B + K_m^b.A.C + K_m^a.B.C + A.B.C}$
3		$v = \frac{(K_1.A.B.C + K_2.A.B^2.C)\ E_t}{K_3.A.B + K_4.A.C + K_5.A.B.C + K_6.B.C + K_7.A.B^2 + K_8.A.B^2.C + K_9.B^2.C}$
4		$v = \frac{(K_1.A.B.C + K_2.A.B^2.C + K_3.A.B.C^2)\ E_t}{K_4.A + K_5.A.B + K_6.A.C + K_7.A.B^2 + K_8.A.C^2 + K_9.B.C + K_{10}.A.B.C + K_{11}.A.B.C^2 + K_{12}.A.B.C^2 + K_{13}.B.C^2 + K_{14}.B.C}$
5		$v = \frac{(K_1.A.B.C + K_2.A.B^2.C + K_3.A.B.C^2)\ E_t}{K_4.A.B + K_5.A.C + K_6.A.B^2 + K_7.A.C^2 + K_8.B.C + K_9.B^2.C + K_{10}.B.C^2 + K_{11}.A.B.C + K_{12}.A.B^2.C + K_{13}.A.B.C^2}$

where A is 2-oxoglutarate, B is CoA, C is NAD^+, P is CO_2, Q is succinylCoA and R is NADH(+ H^+).

In fact the latter mechanism contains the entire Sanadi mechanism, with an additional pathway in which NAD^+ can bind prior to CoA.

Mechanism 3 was taken from Hamada et al. (1975). These authors suggested it as a likely explanation of their initial rate results at high NAD^+ concentrations. Analysis of the results reported in this Thesis by Fromm's method indicated that it is highly unlikely to be the mechanism of the reaction under the conditions used by me, but it was included for comparison purposes. It may be that concentrations of NAD^+ at which Hamada et al. (1975) observed this phenomenon were much higher than those employed in these studies.

It should be noted that mechanisms 2 and 4 are unlikely to be true for the 2-oxoglutarate dehydrogenase complex because it is known that each substrate can react with the complex if an element of the complex is in a suitable form (i.e. in the free, succinylated or reduced state)- in the absence of the other two substrates (see Sanadi, 1963). They were included in the analysis because they are kinetically feasible, i.e. they could not be discounted on the basis of the initial rate studies alone.

Using the Simplex procedure, all five mechanisms shown in Fig. IV.viii.7 were fitted to the experimental data with equivalent initial estimates of the unknown kinetic constants. The parameter which was minimized is R_x, where:

$$R_x = \sum_{1}^{120} \left(\frac{v_{calc} - v_{meas}}{v_{calc} + v_{meas}} \right)^2$$

where v_{calc} is the initial velocity calculated according to the given rate equation containing the current estimates of the unknown parameters, and v_{meas} is the corresponding experimental value (Ottaway, 1973). As explained in Chapter III, there is a positive correlation between initial velocity and the variance, which is abolished by using this

weightingfactor (see Figs. III.2 a & b). The first column in Table IV.viii.1 shows the minimum values of R_x achieved by Simplex for the fitting of each mechanism.

Although these results indicate that mechanism 5 has a much lower residual than others, this does not necessarily imply that mechanism 5 has a better fit to the data. Since each equation has a different number of unknown parameters to be fitted, the values of the minimum differences are not directly comparable. This raises the question of how the fit of the rate equations to the data could be examined more efficiently.

A qualitative examination of the correspondence between experimental and theoretical values of initial rates was made using the SYMAP program as described in Chapter III. The three maps produced for each mechanism give a good visual indication of the overall fit of an equation to the data. The five sets of maps obtained are shown as Figs. IV.viii.8-12. They show that mechanism 5 has few areas of high (>20%) error, and overall has a fairly even correspondence between theoretical and experimental values. The other four mechanisms however, all show areas of very high and very low error interspersed one with another. This visual analysis confirms that mechanism 5 gives the best <u>overall</u> fit of those examined.

The fits were examined statistically in two ways, by a 'Comparison of Models' test and by an Analysis of Variance study. These techniques were suggested by Dr. R.A. Elton of the Department of Medical Statistics, University of Edinburgh; fuller details of the use and reliability of these tests are given by McMinn & Ottaway (1977).

In the 'Comparison of Models' test, a general model (mechanism) is tested against a more specific one, which is a 'special' case of the first model (i.e. the equation for the second model contains all the terms in the equation for the first model plus one or more extra terms). Thus mechanism 1 is a general model for each of the other four, and the

TABLE IV.viii.1. Results of the 'comparison of models' test.

Mechanism	$\underline{R}_m$	d.f.	Mechanisms compared	$\underline{F}_{calc.}$	$\underline{F}$ (0.01)
1	1.798	113			
			1 < 2	4.875	6.84
2	1.723	112			
			1 < 3	1.203	3.17
3	1.722	109			
			2 < 4	0.73	2.55
4	1.631	104			
			1 < 5	14.38	2.55
5	0.858	105			

d.f. is the value of the degrees of freedom calculated from the formula

d.f. = no. of experimental points - no. of parameters being fitted - 1.

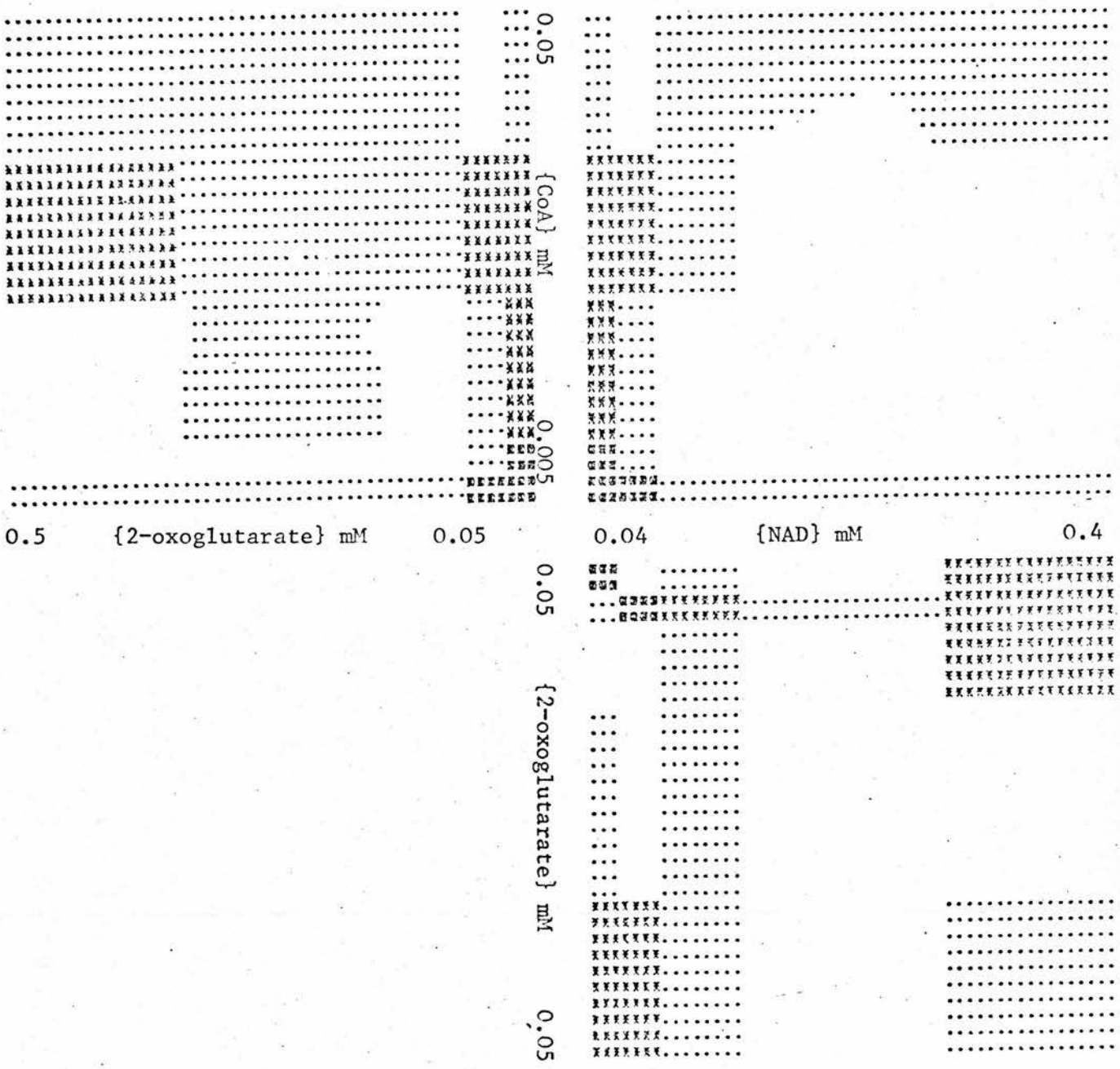
$\underline{R}_m$ is the computed minimum differences according to eqn. 1 in the text. Mechanisms are numbered as in Fig. 2.

F is calculated from the formula

$$\frac{\underline{R}_{m1} - \underline{R}_{m2}}{d_{f1} - d_{f2}} \div \frac{\underline{R}_{m2}}{d_{f2}}$$

$\underline{F}$ (0.01) values for the nearest number of degrees of freedom (d.f.) are taken from Documenta Geigy.

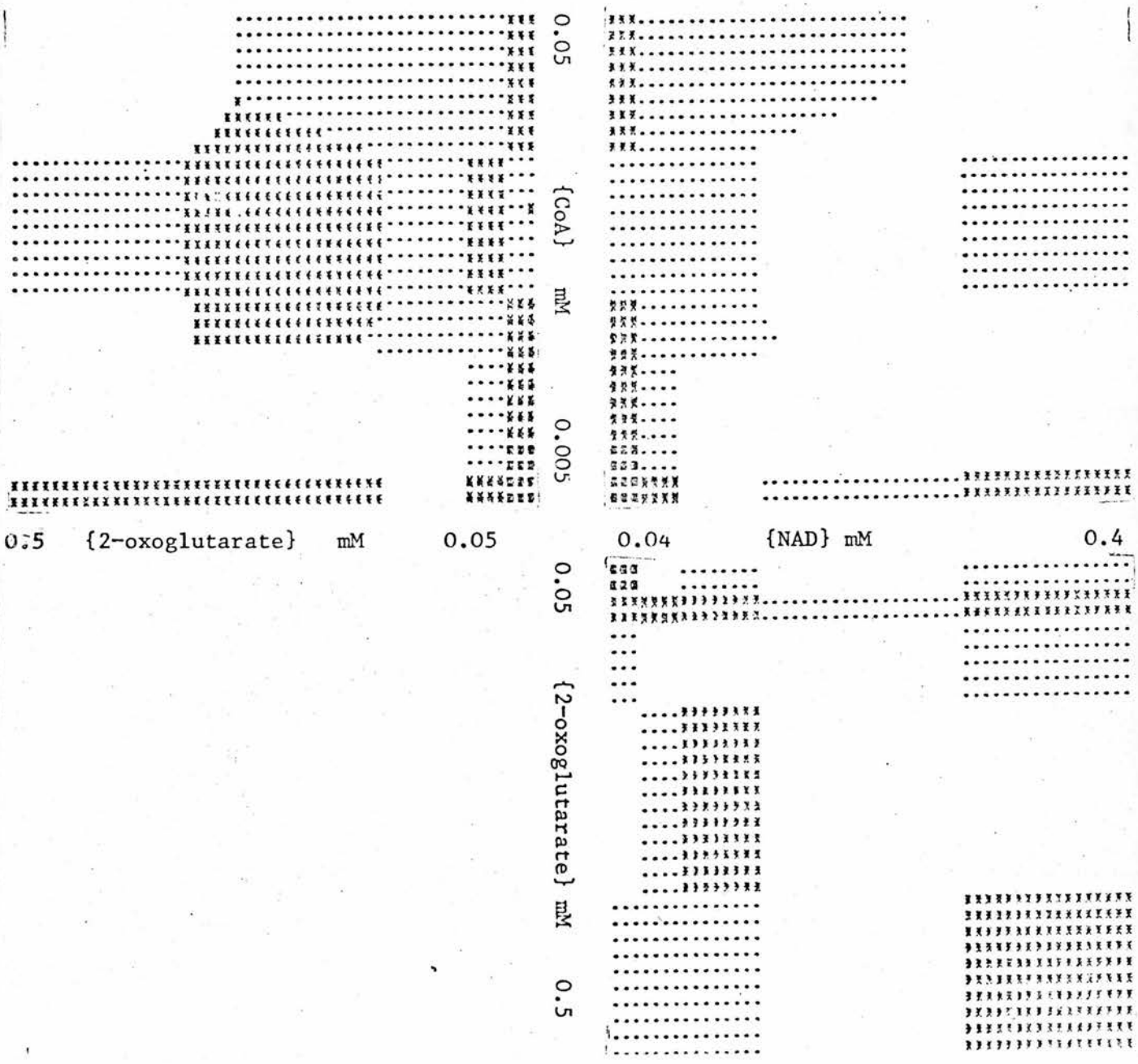
FIG.IV.viii.8. SYMAP representation of the fitting of the Hexa Uni Ping Pong mechanism to the initial velocity data. Percentage error is plotted against substrate concentrations.



Construction of the 'map' layout is dealt with in Chapter III.

The error levels appearing on the maps are 0-10% (blank); 10-20% (.); 20-40% (*); 40-60% (⁂); 60-100% (■).

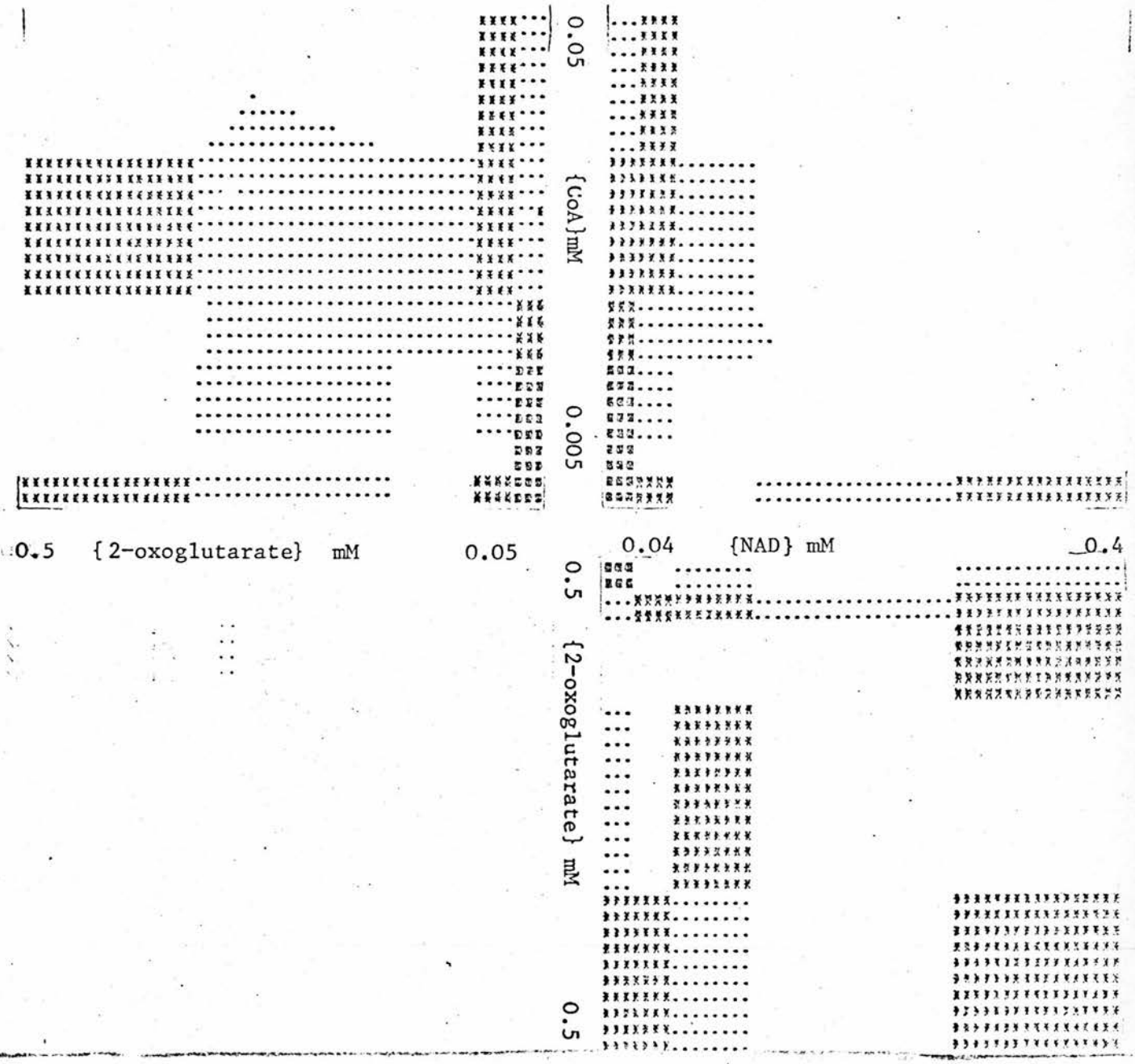
FIG.IV.viii.9. SYMAP representation of the fitting of mechanism 2 to the initial velocity data. Percentage errors are plotted against substrate concentrations.



Construction of the 'map' layout is dealt with in Chapter III.

The error levels appearing on the maps are 0-10% (blank); 10-20% (.); 20-40% (*); 40-60% (米); 60-100% (■).

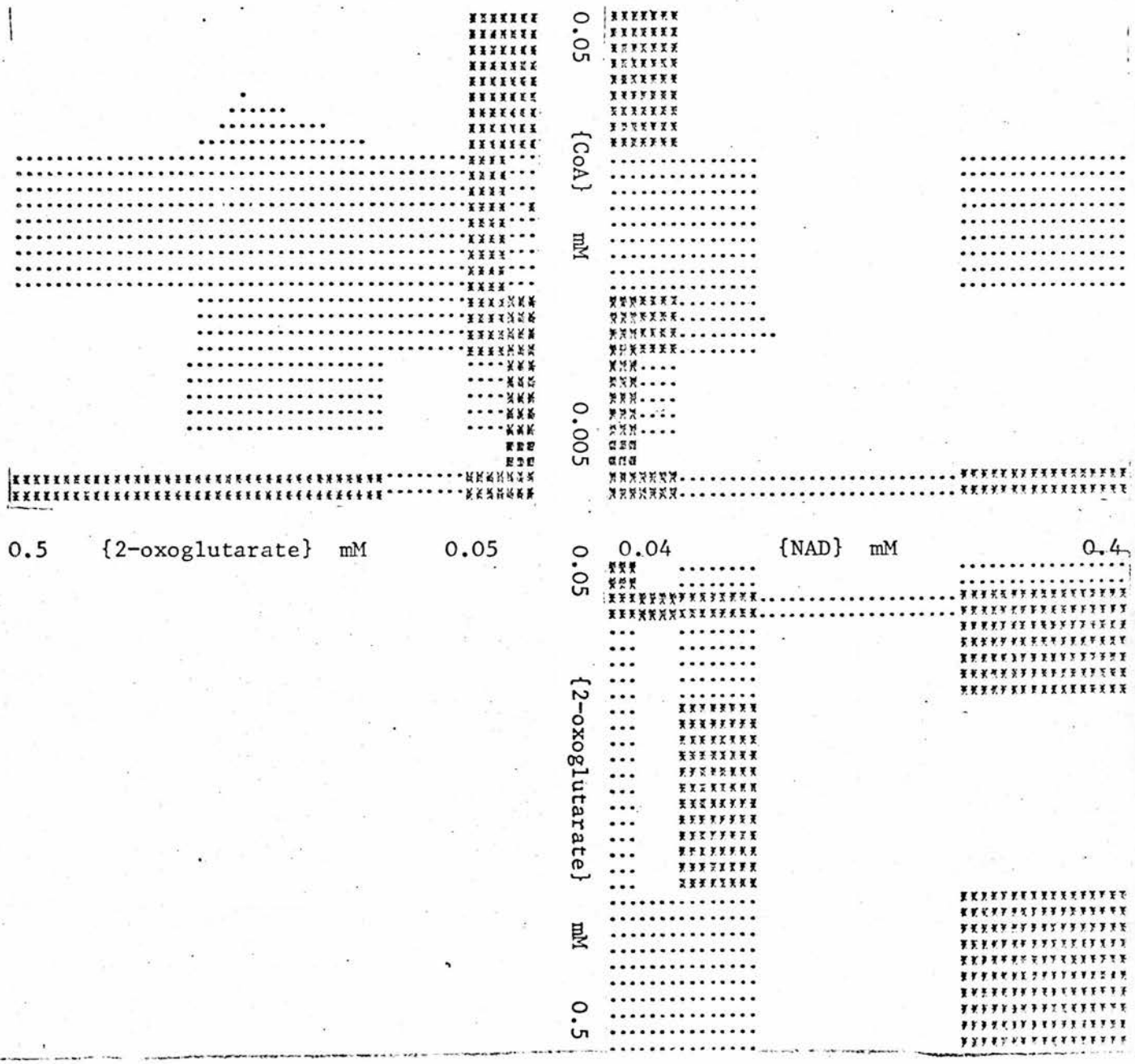
FIG.IV.viii.10. SYMAP representation of the fitting of mechanism 3 to the initial velocity data. Percentage errors plotted against substrate concentrations.



Construction of the 'map' layout is dealt with in Chapter III.

The error levels appearing on the maps are 0-10% (blank); 10-20% (.); 20-40% (*); 40-60% (#); 60-100% (■).

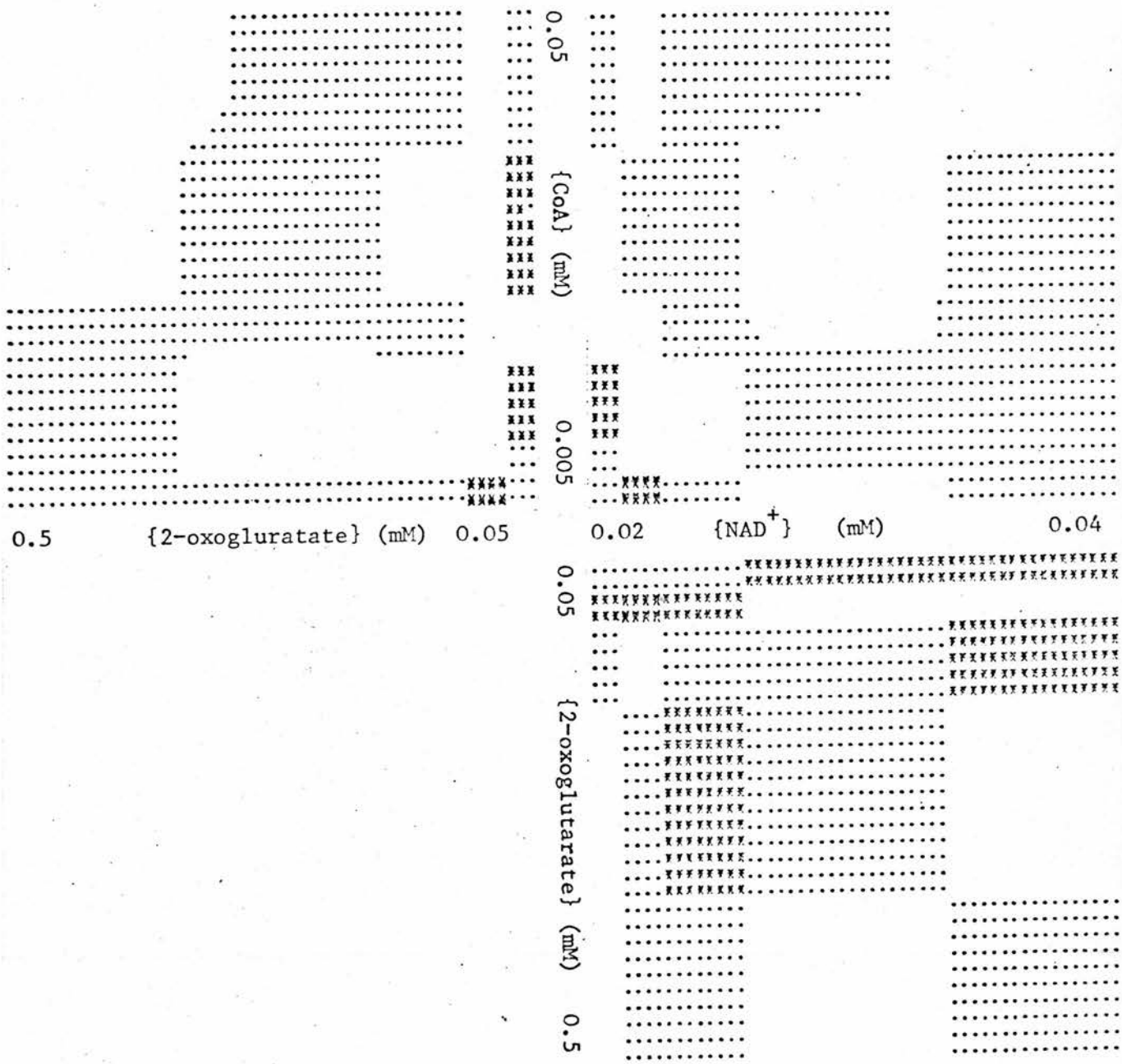
FIG.IV.viii.11. SYMAP representation of the fitting of mechanism 4 to the initial velocity data. Percentage errors are plotted against substrate concentrations.



Concentration of the 'map' layout is dealt with in Chapter III.

The error levels appearing on the maps are 0-10% (blank); 10-20% (.); 20-40% (*); 40-60% (⁑); 60-100% (■).

FIG.IV.viii.12. SYMAP representation of the fitting of mechanism 5 to the initial velocity data. Percentage errors are plotted against substrate concentrations.



Construction of the 'map' layout is described in Chapter III.

The error levels appearing on the maps are 0-10% (blank); 10-20% (.); 20-40% (*); 40-60% (✱); 60-100% (■).

test establishes whether the extra terms in the equations corresponding to the 'special' cases (models 2-5) are contributing to a significantly better fit. The results of this test are shown in Table IV.viii.1. The mechanisms compared are those shown on the third column. The large F value for mechanism 5 can be used as a guide to indicate that this is the only model, of those tested, which gave a better fit to the data than mechanism 1 (Hexa Uni Ping Pong).

The analysis of variance, in simple terms, is an analysis of the origin of the residual difference between experimental and theoretical values of the initial velocities. The residuals consist of two components, one part due to the errors involved in experimental measurements, the other due to the fit between experiment and theory. If a significantly larger part of the minimum difference is contributed by the latter component it is implied that the fit could be improved, i.e., the mechanism does not describe the experimental results satisfactorily. If however, there is no significant difference in the contribution to the residual between the experimental error and the error due to fitting, then the mechanism can be considered as an acceptable fit to the data.

In order to apply this test, that part of the residual due to errors in experimental measurements (R_e) is calculated using the formula:

$$R_e = \tfrac{1}{2} \sum_{i=1}^{60} \left(\frac{v_{i1} - v_{i2}}{v_{i1} + v_{i2}} \right)^2$$

where v_{i1} and v_{i2} are replicate measurements. Using this value of R_e and the value of the minimum differences (the residual, R_m) from the previous tests, the corresponding mean square values were computed from the formulae:

$$MS_m = \frac{R_m - R_e}{df_m - df_e} \qquad \text{and} \qquad MS_e = \frac{R_e}{df_e}$$

where df_m and df_e are the corresponding numbers of degrees of freedom associated with the parameters R_m and R_e respectively.

These mean square values are compared by an 'F' test where

$$F_{((df_m - df_e),\ df_e)} = \frac{MS_m}{MS_e}$$

If the ratio F is significantly high, the error due to the fitting is significantly larger than the error in the experimental measurements. A non-significant value of F indicates no difference between the contribution to the minimum residual of the experimental errors or the errors due to fitting. As previously explained only the latter situation indicates an acceptable fit of the mechanism to the initial rate data. The results of this test are shown in Table IV.viii.2. Of the five mechanisms fitted, only 5 yields a non-significant value for F, inferring that this mechanism has a high probability of being an acceptable description of the experimental data. However as has been pointed out in more detail elsewhere (McMinn & Ottaway, 1977), statistical tests cannot show that any particular mechanism is a unique description of the data. In this study one mechanism does appear as a much better fit than the others, but this does not preclude the possibility that mechanisms other than those tested here may fit equally as well as mechanism 5.

Inhibition Studies with NADH

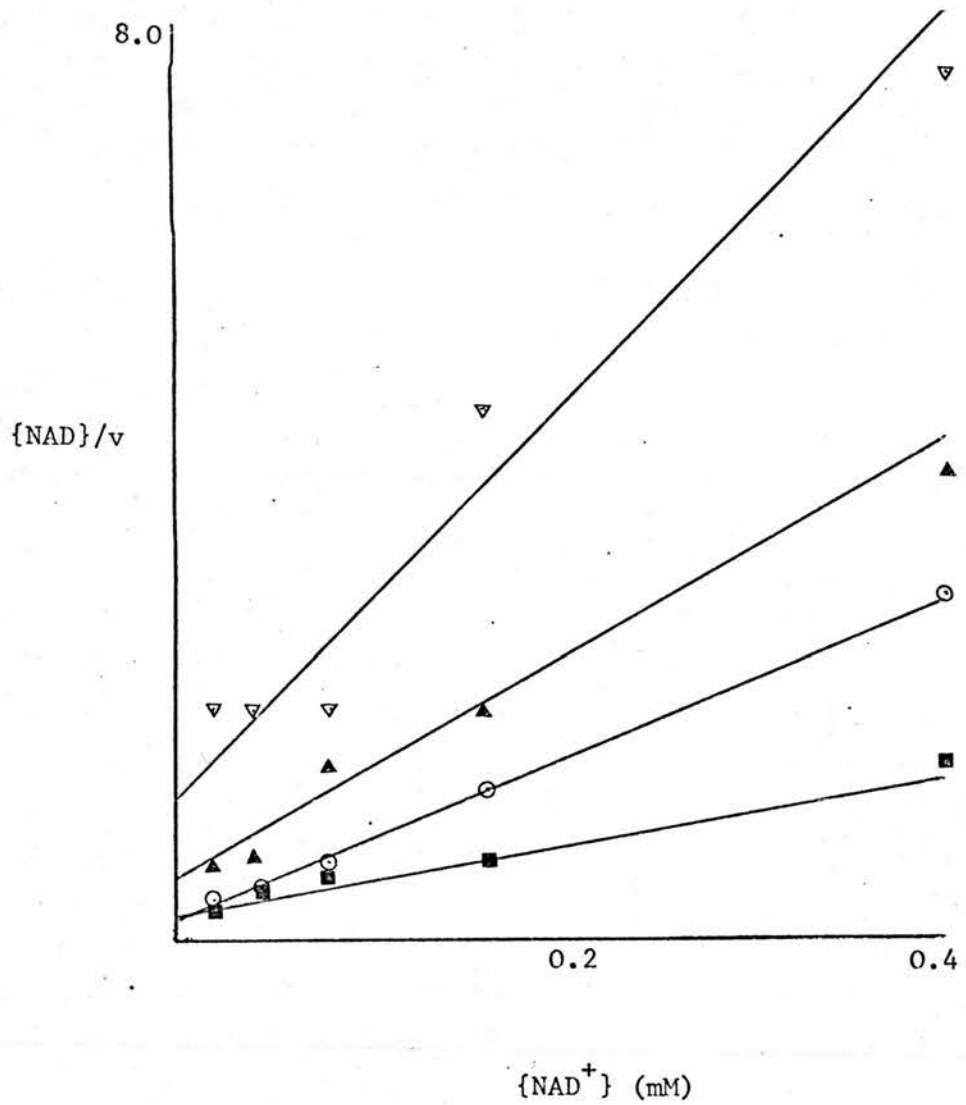
The $\underline{s}/\underline{v}$ against $\underline{s}$ plots of the NADH inhibition results are shown in Figs. IV.viii.13 and 14. Where CoA and 2-oxoglutarate are saturating, the patterns indicate a mixed type inhibition, and this also seems the inhibition type when both these substrates are non-saturating. Straight lines have been fitted to the data, but it should be remembered that these may well be non-linear plots (see Fig. IV.viii.14). Mechanism 5 predicts that the NADH inhibition data should yield non-linear plots, however at the substrate concentrations used, this deviation from linearity is very slight.

TABLE IV.viii.2. Results of the 'analysis of variance' test.

Mechanism	$\underline{R}_m$	d.f.	$\underline{R}_e$	d.f.	MS_m	MS_e	$\underline{F}$
1	1.798	113	0.4064	58	0.0253	0.00697	3.63
2	1.723	112	0.4064	58	0.0244	0.00697	3.50
3	1.722	109	0.4064	58	0.0258	0.00697	3.70
4	1.631	104	0.4064	58	0.0266	0.0069/	3.82
5	0.858	105	0.4064	58	0.0096	0.00697	1.37

Mechanisms are numbered as in Fig. 3. $\underline{R}_m$, $d.f._m$, $\underline{R}_e$, $d.f._e$, MS_m, MS_e and $\underline{F}$ are calculated as described in the text. The theoretical value of $\underline{F}_{50.60}$ in 1.88. This is taken from Documenta Geigy.

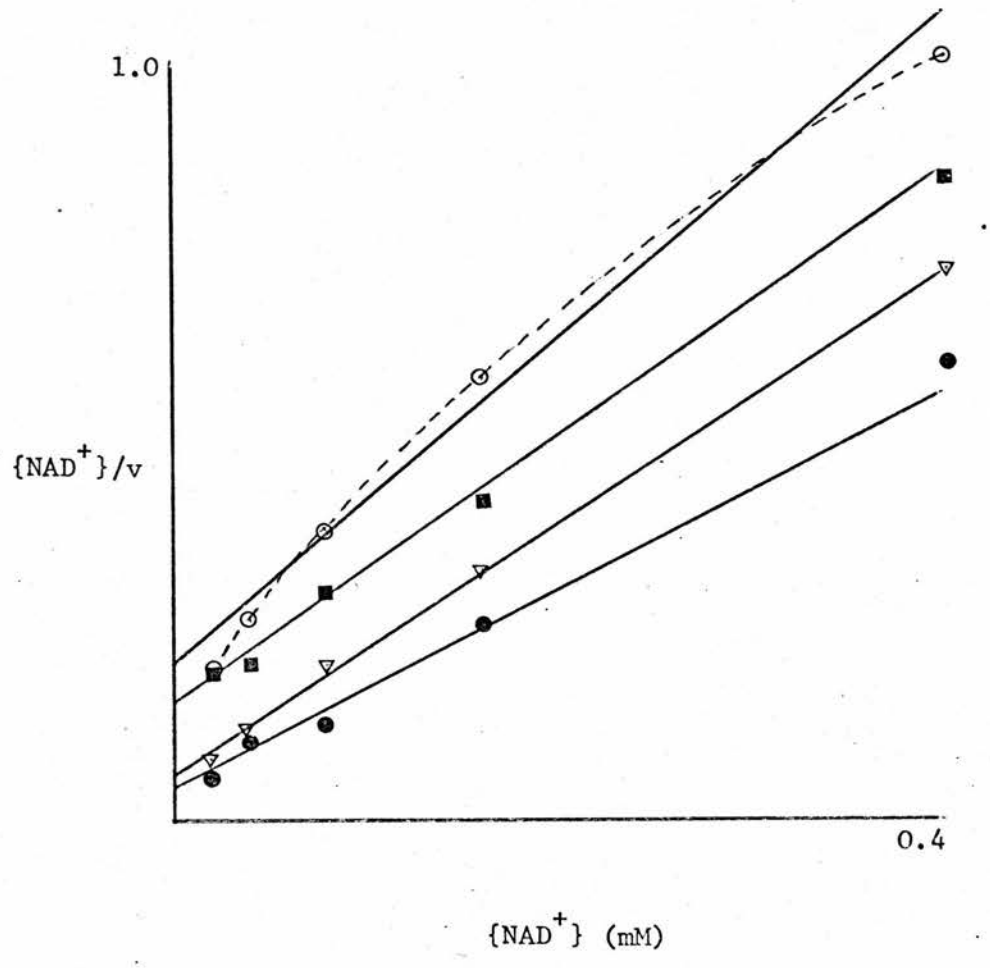
FIG.IV.viii.13. NADH inhibition study of 2-oxoglutarate dehydrogenase: NAD^+ is the variable substrate, CoA and 2-oxoglutarate are at non-saturating concentrations.



All points are the average of two measurements. Velocities expressed as μmoles NADH produced/min/mg protein NADH concentrations are: 0 (■), 0.01mM (○), 0.02mM (▲), 0.05mM (▽). 2-Oxoglutarate concentration is 0.05mM, CoA concentration is 0.005mM.

Lines are fitted by computer as described in Chapter III.

FIG.IV.viii.14. NADH inhibition study of 2-oxoglutarate dehydrogenase: NAD^+ is the variable substrate, CoA and 2-oxoglutarate at saturating concentrations.



All points on the graph are the average of two measurements. Velocities are expressed as μmoles NADH/min/mg protein. Concentrations of NADH are: 0 (●), 0.01mM (▽), 0.02mM (■) and 0.05mM (○). Concentration of 2-oxoglutarate is 0.5mM and of CoA is 0.05mM in all studies. Solid lines are fitted by computer, stippled line fitted by eye.

With CoA as the variable substrate and saturating concentrations of NAD^+ and 2-oxoglutarate, the NADH inhibition appears to be uncompetitive with CoA (see Fig. IV.viii.15). This result agrees with all five mechanisms tested. When 2-oxoglutarate is the variable substrate, the resulting s/v against s plots are non-linear as shown in Fig. IV.viii.16. Although it is obvious that NADH is inhibiting the enzyme reaction under these conditions in some way, it is impossible to analyse such non-linear data easily.

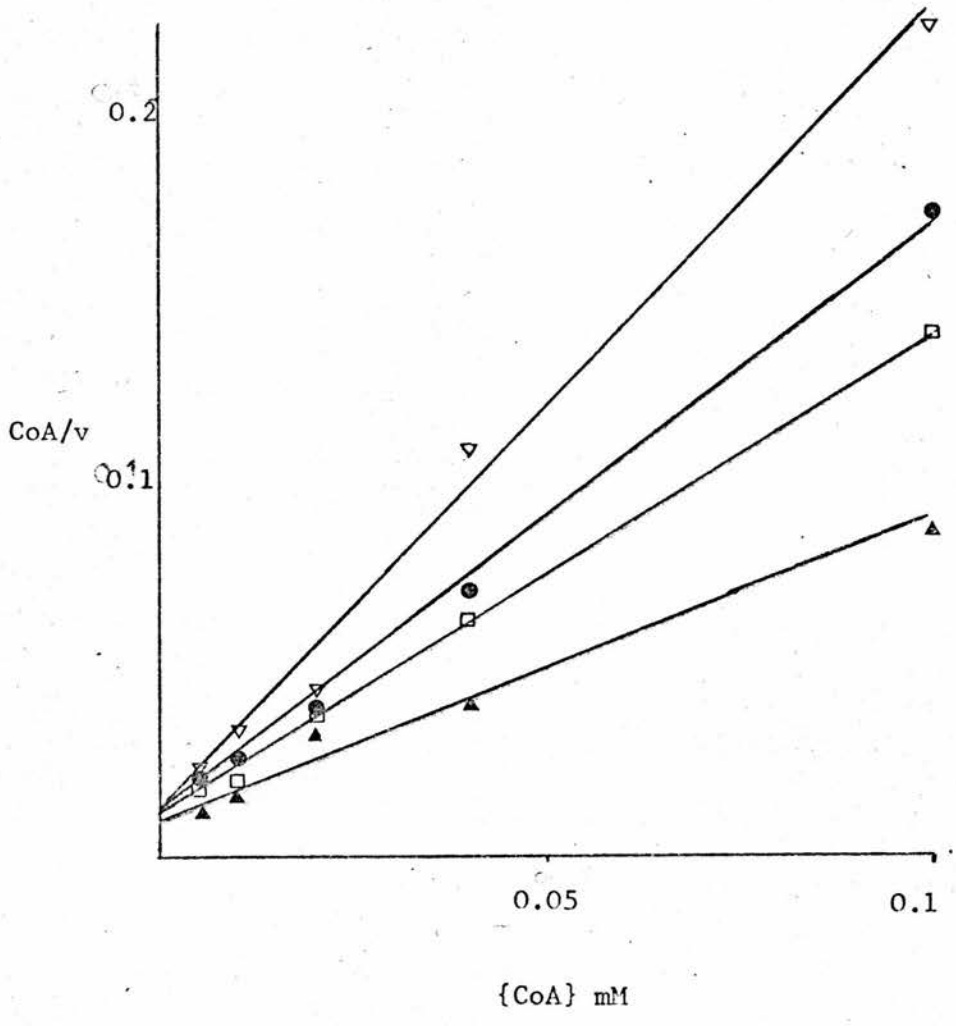
These inhibition studies proved to be of only limited value in establishing a mechanism for 2-oxoglutarate dehydrogenase. They go some way to supporting the premiss that the Sanadi mechanism is not an accurate description of the enzymic reaction, but no further conclusions could be drawn from them. A more complete study, using succinylCoA as an inhibitor, might have yielded sufficient reliable data to be used for a 'total fit' study to be carried out, but neither time nor resources were available for such a large-scale operation to be performed.

Values of Kinetic Constants

The values found by optimisation for the complex constants for Mechanism 5, which were used in subsequent simulation studies, are given in Table IV.viii.3. K_m values have no meaning for rate equations as complex as this, but the 'S_{50}' values for each of the substrates was computed from the values in Table IV.viii.3 by setting the other two substrates at 'saturating' levels. Table IV.viii.4 shows these computed 'S_{50}' values along with the values of K_m for the various substrates which have been reported by previous workers. The agreement is generally good.

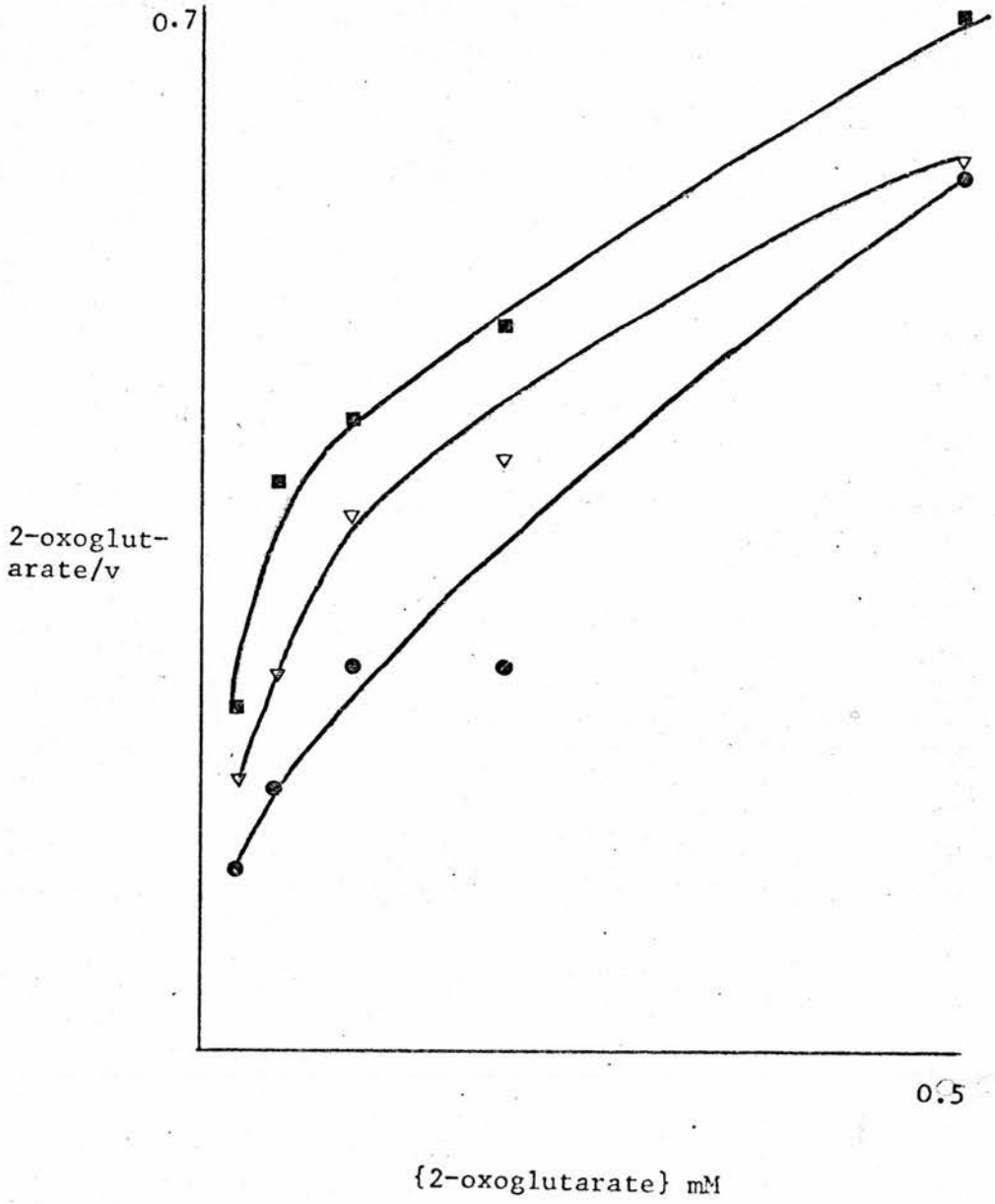
A number of SIMPLEX fittings for mechanism 5 were carried out each using different initial estimates for the complex constants of the rate

FIG.IV.viii.15. NADH inhibition study of 2-oxoglutarate dehydrogenase: CoA is the variable substrate, NAD^+ and 2-oxoglutarate are at saturating concentrations.



Each point indicated on the graph is the average of two measurements. Velocities are expressed as μmoles NADH produced /min/mg protein. Concentrations of NADH used are 0 (▲), 0.01mM (□), 0.02mM (●) and 0.05mM (▽). Concentrations of the fixed substrates are 2-oxoglutarate 0.5mM and NAD^+ 0.4mM. Lines are fitted by computer.

FIG.IV.viii.16. NADH inhibition study of 2-oxoglutarate dehydrogenase; 2-oxoglutarate is the variable substrate, CoA and NAD^+ are at saturating concentrations.



Each point indicated on the graph is the average of two measurements. Velocities are expressed as μmoles NADH produced /min/mg protein. NADH concentrations used are 0 (●), 0.01mM (▽), 0.02mM (■). Concentrations of the fixed substrates are CoA, 0.05mM and NAD^+ 0.4mM. Lines fitted by eye.

TABLE IV.viii.3. Values for the optimized constants of the rate equations for mechanism 5.

Constant	Value
K_1	$21.6s^{-1}$
K_2	$467.0s^{-1}\ mM^{-1}$
K_3	$1703.0s^{-1}\ mM^{-1}$
K_4	$0.75x10^{-10}$ mM
K_5	$0.5x10^{-5}$ mM
K_6	0.44
K_7	0.17
K_8	0.62 mM
K_9	0.20
K_{10}	5.18
K_{11}	$17.8\ mM^{-1}$
K_{12}	$27.1\ mM^{-1}$

The constants are numbered as in mechanism 5 in Scheme 2. Values were calculated in μmol of NADH produced/μmol of enzyme, assuming a mol.wt. of $2.7x10^6$ for the 2-oxoglutarate dehydrogenase complex.

TABLE IV.viii.4. Comparison of kinetic constants and S_{50} values for the pig heart 2-oxoglutarate dehydrogenase.

K_m^{OG} (mM)	K_m^{CoA} (mM)	$K_m^{NAD^+}$ (mM)	Conditions	Reference
0.013	0.0001	0.0045	pH 7.4 25°C	Massey (1960)
0.22	0.025	0.05	pH 7.5 25°C	Hamada et al. (1975)
---	0.0027	0.021	pH 7.2 22°C	Smith et al. (1974)
S_{50} OG (mM)	S_{50}CoA (mM)	$S_{50}NAD^+$ (mM)		
0.2	0.0035	0.05	pH 7.2 30°C	The present study

S_{50} values are calculated as described in the text. The S_{50} value given here is the lowest concentration of CoA that will give half-maximal velocity. Mechanism 5 predicts that CoA will exhibit substrate inhibition such that there is more than one concentration of CoA that can give half-maximal velocity.

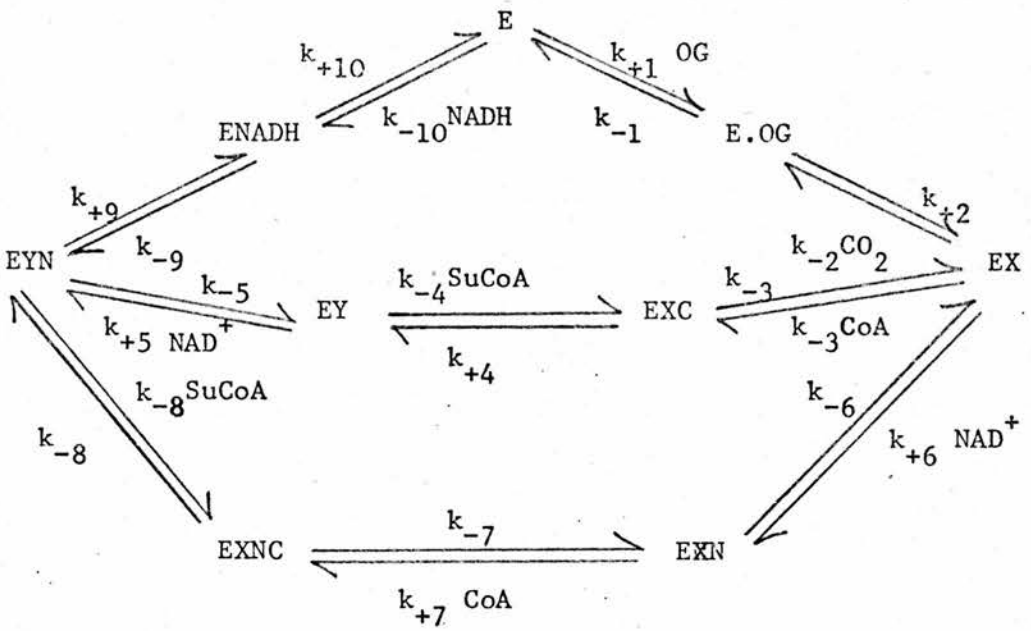
equation. They all reached a similar minimum value for the sum of the deviations, but with different final values for the complex constants, i.e., there is no global minimum for the optimization. (This was not repeated for all five mechanisms because of the expense of the computer running time for such large optimizations, since mechanism 5 gave a much better fit than the others, using a single set of starting values for each of the five models.)

Part of the purpose of this work was to find values of the rate constants for each reaction step of the enzymic mechanism, so an attempt was made to fit values for the twenty individual rate constants of mechanism 5 to the optimized complex constants for this mechanism. The initial equation generated contains thirteen complex constants. These can be reduced to twelve by dividing throughout by K_{11} in the equation shown in Fig. IV.viii.7, and this is a more correct representation of the rate equation for statistical comparison with mechanism 1. However, for purposes of evaluation of individual rate constants it is much simpler to take optimized values for thirteen complex constants and resolve these, since each contains fewer rate constants per complex constant. It then becomes much easier to fit a few rate constants at a time to the complex constants in which they appear.

None of the sets of complex constants found by repeated optimizations led to a complete set of internally consistent step rate constants. The rate constants k_{-2}; k_{-4}; k_{-8}; k_{-10}; do not appear in the initial rate equation when products are omitted from the assay system, so these could not be determined in any case). Using the values given in Table IV.viii.3, values for the sixteen valid rate constants could be found which fit eleven of the complex constants almost exactly and fit to the remaining two complex constants (K1 and K8) very badly. The fitting was performed by taking the rate constants, k_{+1}, k_{+2}, k_{-3}, k_{+4},

k_{+5}, k_{+6}, k_{+7}, k_{-7}, k_{+8}, k_{+9}, k_{-9}, k_{+10}, and fitting them to the complex constants containing only these rate constants, which are K3, K7, K10, K13. An exact fit could be found between these 12 rate constants and this subset of the complex constants. Then the complex constants which contain k_{+3} were added (K2, K6, K9, K12) and the optimization was repeated, starting with the optimized values for the twelve rate constants previously optimized. Once again a value for this rate constant could be found such that there was an exact fit between the complex constants and the thirteen rate constants. The three remaining constants k_{-1}, k_{-5}, k_{-6} were treated in a similar way. At this point the optimization did not reach a satisfactory minimum, and even in the best fit that could be obtained, only eleven of the thirteen complex constants could be fitted. The rate constants obtained from this optimization are shown in Table IV.viii.5. The constants which seem to be least compatible with the complex constants are k_{-5} and k_{-6}, both of which are contained in the random part of the reaction. This indicates that, as previously suggested, mechanism 5 is not a completely accurate description of the 2-oxoglutarate dehydrogenase system, and that the inadequacy lies at the branching points of the random pathway. However, the number of possible changes, such as irreversible steps, interconversions of more complex binding sequences, which could be made to the mechanism is great and the calculation of rate equations and the fit to the experimental data for each of these would be an almost impossible task. It was decided to take the values of the rate constants found by optimization and use them for simulation. Values for the four unobtainable rate constants (k_{-2}, k_{-4}, k_{-8}, k_{-10}) were calculated from the value of the equilibrium constant. For this mechanism the relationship is:

TABLE IV.viii.5. Mechanism and Optimized Rate Constants for the improved Model of 2-Oxoglutarate Dehydrogenase.



Optimized Values of Rate Constants

Constant	Value	Constant	Value
k_{+1}	3.51×10^{2} $mM^{-1}sec^{-1}$	k_{-1}	1.22×10^{2} sec^{-1}
k_{+2}	2.002×10^{3} sec^{-1}	k_{-2}	2.6×10^{-1} $mM^{-1}sec^{-1}$
k_{+3}	6.58×10^{2} $mM^{-1}sec^{-1}$	k_{-3}	1.88×10^{4} sec^{-1}
k_{+4}	3.12×10^{4} sec^{-1}	k_{-4}	2.6 $mM^{-1}sec^{-1}$
k_{+5}	8.58×10^{2} $mM^{-1}sec^{-1}$	k_{-5}	$2.18 \times 10^{+1}$ sec^{-1}
k_{+6}	1.768×10^{3} $mM^{-1}sec^{-1}$	k_{-6}	8.06×10^{-1} sec^{-1}
k_{+7}	3.08×10^{5} $mM^{-1}sec^{-1}$	k_{-7}	3.07×10^{4} sec^{-1}
k_{+8}	1.144×10^{3} sec^{-1}	k_{-8}	1.534×10^{3} $mM^{-1}sec^{-1}$
k_{+9}	1.82×10^{2} sec^{-1}	k_{-9}	2.09×10^{6} sec^{-1}
k_{+10}	1.06×10^{6} sec^{-1}	k_{-10}	4.46×10^{4} $mM^{-1}sec^{-1}$

Where OG is 2-oxoglutarate and SuCoA is succinylCoA. Rate constants are calculated as described in the text.

$$K_{eq} = \frac{k_{+1}\ k_{+2}\ k_{+3}\ k_{+4}\ k_{+5}\ k_{+9}\ k_{+10}}{k_{-1}\ k_{-2}\ k_{-3}\ k_{-4}\ k_{-5}\ k_{-9}\ k_{-10}}$$

$$= \frac{k_{+1}\ k_{+3}\ k_{+6}\ k_{+7}\ k_{+8}\ k_{+9}\ k_{+10}}{k_{-1}\ k_{-3}\ k_{-6}\ k_{-7}\ k_{-8}\ k_{-9}\ k_{-10}}$$

The arbitrary assumptions were made that k_{-2} (the binding of CO_2 to the enzyme-TPP-succinyl form) is very small, and that k_{-10} (the binding of NADH to the free enzyme) is relatively large, and values for the four rate constants were calculated to fit with the value of the K_{eq} of 0.75×10^6. These are shown also in Table IV.viii.5.

Discussion

The conclusions arrived at in this study not only disagree with the mechanism proposed by Sanadi, they also disagree with the findings of Hamada et al. (1975). Initial rate studies carried out by these latter authors supported the Sanadi mechanism in general, though the evidence suggested that this mechanism does not hold at high NAD^+ concentrations.

It is possible that the non-linearity of the reciprocal plots could be due to homotropic allosteric effects but there is at present no evidence for allosteric behaviour of the 2-oxoglutarate dehydrogenase system, in contrast with the pyruvate dehydrogenase system of E. coli, for which binding of acetylCoA at sites other than catalytic sites has been established (Shepherd & Hammes, 1976).

It should be emphasized again that mechanism 5 may only be the simplest one which gives an acceptable fit to the data. It has been assumed in constructing the models shown in Fig. IV.viii.7, that the binding of 2-oxoglutarate and the release of CO_2 are the first and obligatorysteps in the catalysis, preceding the other two stages of the

reaction, and that the release of NADH is the last step in the reaction mechanism, but this need not be so. The NADH inhibition plots (Figs. IV.viii.15-18) are not simple; moreover the distribution of error, even on the best model, is worse in relation to NAD^+ than to the other two substrates (Fig. IV.viii.14). These findings together with the results of Hamada et al. (1975), suggest that the behaviour of the enzyme towards NAD^+ (or NADH) is not straightforward. A more complicated sequence of intramolecular transfers could be envisaged which could describe the sequence of events at this point in the reaction more accurately.

Should a more complex mechanism be designed, the problem then arises of distinguishing the improved fit of such a model over mechanism 5. The latter is already significant at the 1% level in the Analysis of Variance test; more complex models might give an equally significant result, and there would be no criterion for choosing between them. It is possible that the 'Comparison of Models' test could be used to distinguish an improved fit to the data but more complex models would involve more parameters in the rate equation, and the reduction in the residual error returned by the optimization program would have to be large before statistical significance could be attained. Analysis of more complex models would also involve a number of problems concerning the physical handling of such mechanisms. Both the construction of the rate equations and the optimization procedure would be very time-consuming.

One aspect of 2-oxoglutarate dehydrogenase which is rarely taken into account in a discussion of its kinetic behaviour, is the physical structure of the enzyme. It has long been established that it is a complex of three different types of subunit (Reed, 1966; Koike et al., 1971). In all kinetic studies on the enzyme reported by other authors, as indeed in this study, it has been assumed that the enzyme behaves as if only three peptide chains are involved, one for each partial reaction.

The number of each of the three types of polypeptides within the complex is not known exactly, although Tanaka et al. (1972) have suggested that the ratio of decarboxylase, transuccinylase and lipoate dehydrogenase chains is 6:1:6. The implicit assumption is that this ratio confers roughtly equal catalytic power to the three types of subunit. There is no evidence to contradict this assumption (see McMinn & Ottaway, 1977). However the architecture of the enzyme complex is such that there is a large number of each type of subunit arranged in a geometric array (Koike et al., 1971). It may be that each type of subunit can react with more than one of the protein chains catalysing the neighbouring partial reaction of the sequence shown in Fig. IV.viii.1. There is certainly no evidence to suggest that this is not the case. It seems likely that the ability of each subunit to react with more than one of another type is the basis of the observed semi-random character of the reaction mechanism.

As previously explained, it is assumed that the transfer of a succinyl residue and $2H^+$ from the decarboxylase subunit to a transuccinylase subunit is necessary before subsequent reactions can proceed. If the complex as a whole is not working at maximum activity, it is possible that the transuccinylase subunit which picks up the succinyl residue is not the one which accepted the previous residue. This subunit could then remain in the succinylated form for some time before being regenerated, so long as there remains a pool of unsuccinylated subunits available to accept succinyl residues from the decarboxylase subunit. A similar situation could be argued for the dehydrogenase subunits. This condition may be viewed as a type of 'Dead End' inhibition where a number of transuccinylase or dehydrogenase subunits are not immediately required for catalysis, but may exist in the substituted form for some time.

Both random binding of substrates and 'Dead End' inhibition produce rate equations which contain squared substrate terms in the numerator and denominator. However, it is difficult to differentiate kinetically between the two situations (c.f. Feraudi et al., 1977).

This situation can only be visualized as existing so long as substrate concentrations are non-saturating, i.e. the complex is operating below maximum activity. The Ping Pong nature of the reaction observed in previous kinetic studies of 2-oxoglutarate dehydrogenase by Massey (1960) and by Hamada et al. (1975) obviously describes the situation occurring when the enzyme is operating at maximal activity. It is accounted for by Mechanism 5, since if all the subunits are in the substituted state, the release of a product molecule is necessary before further reaction can take place, hence the mechanism becomes Ping Pong.

If the observed random character of the enzymic reaction is due in some way to the molecular architecture of the enzyme complex, a more accurate correlation between enzyme structure and catalysis in a rate law (e.g., by representing >1 active sites per enzyme molecule) may lead to a more accurate analysis of the initial rate data, and hence to a completely acceptable enzymic mechanism. This must remain as a sought-after ideal until the exact architecture of the enzyme complex is published.

Although mechanism 5 may not be a complete description of the mechanism of 2-oxoglutarate dehydrogenase, the work which has been described shows clearly that it is an improvement on the Sanadi mechanism (Hexa Uni Ping Pong), and it is certainly more useful for the simulation study.
There is no reason to assume that the substrates of the enzyme are at saturating concentrations in the mitochondria under all conditions, and thus the random character of the enzymic mechanism could be important to Cycle control, particularly with respect to alterations in the steady state levels of CoA and NAD^+.

Mechanism 5, using the rate constants in Table IV.viii.5 was included in the Citric Acid Cycle simulation in place of the original equation deck which contained the Sanadi mechanism. The results of this work are reported in the following section.

Section ix. Simulation Studies with the Citric Acid Cycle Model containing the Random Mechanism for 2-Oxoglutarate Dehydrogenase

A simulation model of the Cycle containing the second equation deck for 2-oxoglutarate dehydrogenase was set up with fixed ratios of NAD^+:NADH, acetylCoA : free CoA, ATP:ADP and GTP:GDP as in previous simulations. This attained a steady state which differs in a number of respects from the earlier version of the Cycle. The principal differences are the steady-state concentration of 2-oxoglutarate, and the Cycle flux. The free concentrations of all the other intermediates and coenzyme species were reduced to some extent. A comparison of the steady state with that for the simulation containing the Sanadi mechanism is shown in Table IV.ix.1. The decreased flux is due to the decrease in {oxalacetate}. The decrease in concentration of the free pyridine nucleotides is due to binding (particularly of NADH) to 2-oxoglutarate dehydrogenase; this was not possible with the Sanadi mechanism. (The decrease in free $\{NAD^+\}$, although much less in percentage terms, than that of oxalacetate, also contributes to the slowing of the Cycle.)

The Sensitivities and Elasticities of the Cycle enzymes were compared with those produced by the previous simulation model. The Sensitivities of the important Cycle enzymes were measured as before. Elasticities of the NADH-linked dehydrogenases towards NADH were measured for these enzymes in isolation only, since previous studies indicated that this gives the same results as measurement of the Elasticities within the Cycle. The results are shown in Table IV.ix.2. Comparison with Tables IV.i.2.

TABLE IV.ix.1. Comparison of the steady state concentrations for citric acid cycle simulations containing two different models for 2-oxoglutarate dehydrogenase.

Parameter	Model with Sanadi mechanism	Model with Random mechanism
AcetylCoA	3.3×10^{-2}	3.3×10^{-2}
Citrate	2.179×10^{-2}	1.9911×10^{-2}
cis-Aconitate	8.362×10^{-4}	7.6422×10^{-4}
Isocitrate	1.337×10^{-3}	1.222×10^{-3}
2-Oxoglutarate	7.9079×10^{-6}	5.9501×10^{-2}
SuccinylCoA	2.1957×10^{-3}	1.9955×10^{-3}
Succinate	2.7742×10^{-2}	2.4789×10^{-2}
Fumarate	9.655×10^{-2}	8.7648×10^{-2}
Malate	4.8952×10^{-1}	4.4439×10^{-1}
Oxalacetate	6.2098×10^{-5}	5.6406×10^{-5}
CoA	6.6×10^{-2}	6.6×10^{-2}
NAD	1.7191×10^{-1}	1.6959×10^{-1}
NADH	1.9386×10^{-1}	1.9124×10^{-1}
Flux at Steady State	1.1969×10^{-3}	1.0899×10^{-3}
NAD^+:NADH	0.887	0.887
AcetylCoA:free CoA	0.5	0.5

Ratios or ATP:ADP, GTP:GDP and the concentration or P_i are all fixed at the values used in Sections i-v. of this Chapter.

Values outlined are fixed ratios.

TABLE IV.ix.2. Sensitivities and elasticities (to NADH) of the citric acid cycle enzymes with the random version of the 2-oxoglutarate dehydrogenase mechanism.

Enzyme	Sensitivity	Elasticity to NADH
Citrate synthase	0.872	-
Isocitrate dehydrogenase	0.064	-0.76
2-oxoglutarate "	0.092	-0.99
Succinate dehydrogenase	0.046	-
Malate dehydrogenase	0.037	-37.5

Simulations carried out with fixed ratios of NAD^+:NADH and acetylCoA:free CoA.

and IV.i.3.shows that there are no major changes in any of the values. 2-oxoglutarate dehydrogenase now has a slight Sensitivity and Elasticity, but under the conditions employed the values are not of great significance, and make no difference to the conclusions about the control of the Cycle made from the earlier studies.

The Sensitivities of the Cycle enzymes with the random mechanism for 2-oxoglutarate dehydrogenase, under conditions of floating acetylCoA: free CoA and NAD^+:NADH ratios were also examined. These are shown along with the equivalent values for the first Cycle model in Table IV.ix.3. The general pattern of results is very similar in both cases with 2-oxoglutarate dehydrogenase showing a small positive Sensitivity in the second simulation.

These studies indicate that, although the random mechanism is an improved description of the modus operandi of 2-oxoglutarate dehydrogenase, under the conditions employed for the simulation studies the enzyme does not play an important role in the control of flux through the Citric Acid Cycle. The fact that the improved model of the enzyme does exhibit a positive Sensitivity value suggests that there may be conditions other than those investigated here, in which it may become a more important control point for Cycle flux.

TABLE.IV.ix.3. Sensitivities of the citric acid cycle enzymes for the two simulation models containing different models for 2-oxoglutarate dehydrogenase.

Enzyme	Model with Sanadi mechanism	Model with Random mechanism
Citrate synthase	0.192	0.184
Isocitrate dehydrogenase	-0.092	-0.083
2-Oxoglutarate dehydrogenase	0.0	0.018
Malate dehydrogenase	-0.100	-0.092
AcetylCoA supplier	0.159	0.156
'ETS'	0.560	0.560

Ratios of NAD^+:NADH and acetylCoA:free CoA are allowed to 'float', (as per Section v. of this Chapter).

CHAPTER V: GENERAL CONCLUSIONS AND DISCUSSION

The results reported in the previous Chapter lead to the general conclusion that, as proposed by Krebs & Lowenstein (1960), the most important control 'point' of the Citric Acid Cycle is at the initiating reaction of the pathway. Although the control is exerted principally at this one site, there are a number of factors involved in the regulation. The most important are the activity of citrate synthase, the rate of production (and hence the steady-state concentration) of acetylCoA and the steady state concentration of oxalacetate. However the parameters which govern these three primary factors, such as the NAD^+:NADH ratio or the flux through glycolysis, also play a part in Citric Acid Cycle regulation through their effects on the primary regulatory factors.

The control of the Cycle flux through the activity of citrate synthase was very clearly demonstrated in sections i. and iii. of the previous Chapter. As explained there the very high affinity of the enzyme for both substrates means that under the conditions designated as 'normal' (i.e. acetylCoA concentration of 0.033 mmol/kg mito. and oxalacetate concentration of 6.21×10^{-5} mmol/kg mito.), the enzyme is virtually saturated with acetylCoA. This has important implications in two respects. First there is very limited capacity to increase flux through the Cycle by increasing the steady-state concentration of acetylCoA, while there is a large capacity to increase flux by increasing the steady-state concentration of oxalacetate. Second, it casts doubt on the physiological significance of proposed 'Regulators' of the activity of the enzyme. SuccinylCoA (LaNoue *et al.*, 1972), NADH and acetoacetylCoA (Srere & Matsuoka, 1972), all show inhibition, competitive with acetylCoA, of citrate synthase in *in vitro* experiments. However the K_i values for these inhibitions are all much higher than the Km value for acetylCoA, implying that they would have very little effect on the enzyme activity unless

present in very high concentrations relative to acetylCoA. Such situations are improbable, especially with acetoacetylCoA, which in the studies in section viii. of Chapter IV fell to a very low steady-state concentration (2.75×10^{-6} mmol/kg mito.). Inhibition by succinylCoA was included in the simulation for all the studies reported in the previous Chapter. Even in the simulations where the succinylCoA concentration increased by about 4-fold as a result of altering the GTP:GDP ratio (section viii), the inhibitory effect on citrate synthase was negligible. (In the 'normal' situation the amount of enzyme in the inhibited form is 0.15% of the total enzyme). Srere (1971) has also cast doubt on the physiological significance of these proposed inhibitors. However LaNoue et al. (1972) suggested that the inhibition of citrate synthase by succinylCoA would become important under conditions of low acetylCoA, and high succinylCoA, concentrations. It is equally possible that the decreased flux under such conditions is entirely due to the decrease in acetylCoA alone and that any succinylCoA bound to the enzyme would be binding to sites that would otherwise be free. Of course this assumes the oxalacetate concentration to be very much lower than that either of acetylCoA or of succinylCoA, a reasonable assumption considering the equilibrium of the reaction catalysed by malate dehydrogenase.

The rate of production and the steady-state concentration of acetylCoA will obviously affect the flux through citrate synthase. As detailed above this has an upper limit above which no further increase in Cycle flux can be elicited due to the saturation of the enzyme. However at lower rates of acetylCoA input the Cycle flux becomes very dependent on the rate of acetylCoA production and little or no control remains with citrate synthase under such conditions.

The steady state concentration of oxalacetate is also of great importance to the flux through citrate synthase, and hence through the Cycle, as explained in section iii of the Chapter IV. Although it is the concentration of this intermediate which regulates Cycle flux, its ability to perform this function is due to the features of the enzyme producing it, that is, malate dehydrogenase. The very low equilibrium constant of the reaction ensures that the concentration of oxalacetate remains very much lower than that of malate. Together with the fact that malate dehydrogenase catalyses an 'equilibrium' reaction, this confers strategic importance on oxalacetate. If it is maintained at a low but steady concentration, oxalacetate 'limits' the flux through citrate synthase. This is normally the case, since even if its concentration should fall temporarily because of removal by other pathways or binding to other enzymes/proteins in the mitochondrial matrix, it will immediately be replenished from the pool of malate via the equilibrium reaction of malate dehydrogenase. Equally important, is the fact that malate dehydrogenase is a pyridine nucleotide-linked enzyme. This means that any change in the ratio of NAD^+:NADH will cause a corresponding change in the oxalacetate concentration and hence the flux through citrate synthase. The concentration of malate is much higher, and hardly changes at all. Through the reaction of malate dehydrogenase there is thus a direct link between Citric Acid Cycle flux and the electron transport chain, and to energy production by oxidative phosphorylation.

These three factors, the equilibrium distribution of the reactants, the 'equilibrium' nature of the reaction and the participation of the pyridine nucleotides in the reaction, although in themselves potentially control features, only become of crucial importance because of the position of malate dehydrogenase within the pathway. If any other enzyme of the pathway had possessed these features, it would have been unlikely to have

commanded such power over the flux through the Cycle. The results of the Elasticity studies in Section i. of the previous Chapter emphasize this with respect to the NAD^+:NADH ratio. Even with a relatively large change in this ratio,there is only a very small change in Cycle flux caused by changes in activity of isocitrate or 2-oxoglutarate dehydrogenases. The changes in Cycle flux that result from changes in the NAD^+:NADH ratio can be almost entirely ascribed to effects on malate dehydrogenase.

The importance of oxalacetate at the junction point of the Cycle, being both substrate and product of the pathway, is all the more important because of its low steady-state concentration. If the concentration of oxalacetate were to lie in the same range as the other Cycle intermediates control of flux through citrate synthase would not be possible. Because of the high affinity of citrate synthase for both its substrates, it would then be completely saturated under almost all conditions, and it would be impossible for any change in the oxalacetate concentration to change the throughput of the enzyme. Only because the concentration of oxalacetate in mitochondria is so very low can delicate and sensitive control of the Cycle flux be exerted at citrate synthase.

Control of the Citric Acid Cycle flux must be considered as a feature of the entire system around the start/finish junction of the Cycle, that is, the combination of malate dehydrogenase and citrate synthase. Both enzymes are critical to this control; the features of each individually are relatively unimportant when considered separately from the other. Together they form a highly sophisticated and powerful control system.

It is perhaps fitting that the importance of such a relationship between two enzymes should first have been suggested by Krebs (1969), when he pointed out that so-called 'equilibrium' enzymes can play an important role in rate control through their ability to maintain and alter substrate concentrations particularly where they precede a 'rate-

controlling' enzyme such as in the case discussed here. These ideas were extended by Rolleston (1972), and this author defined a 'Rate Limiting System' which very closely resembles the situation found in the simulation studies reported here, the entire system around the initiating reaction of the Cycle contributing to the regulation.

One further level of sophistication of the regulation of Citric Acid Cycle by the malate dehydrogenase/citrate synthase system is its unique ability to work in concert with both the pathways supplying oxidizable carbon to the Cycle and the energy-producing pathways which utilize the reducing equivalents generated within the Cycle. Not only can each of these be co-ordinated with the Cycle but through this system they can also be co-ordinated with one another. Since both 'supply' and 'demand' exert their major control at this one point in the Cycle the balancing of Cycle throughput between supply and demand phenomena becomes extremely efficient. There is no wastage or redundancy inherent in any part of the Cycle; either the acetylCoA enters and is completely oxidized or it does not enter at all.

It must be borne in mind, however, that both these pathways of supply and demand will themselves be under extraneous controls and are very probably co-ordinated by many other mechanisms. In this respect the level of acetylCoA reaching the Cycle, and the ratio of NAD^+:NADH in the medium within which the Cycle operates, are under the constraints of a number of complex control systems. On balance, it is probable that control by the Cycle of the pathways which supply acetylCoA and oxidize NADH is small, while the effect of these pathways on the Cycle flux is considerable.

It should be re-iterated at this point that the preceding discussion refers only to the results of this study, that is to the situation in heart muscle. Other considerations would have to be taken into account in other tissues, notably in liver.

The enzymes of the Citric Acid Cycle other than citrate synthase and malate dehydrogenase control the Cycle to a very small extent, except in a few extreme situations. Isocitrate dehydrogenase, although often regarded as a major control point (see Plaut, 1970) showed only a very small Elasticity towards NADH and a negative or zero Sensitivity under most conditions employed for the simulation studies. Only when the ATP concentration was very high (section vi. Chapter IV) did isocitrate dehydrogenase show a positive Sensitivity value. Even then, the Sensitivity was not large relative to the other enzymes in the system. This leads to the conclusion that in heart muscle at least, this enzyme does not control Citric Acid Cycle flux.

In the simulation studies in which 2-oxoglutarate dehydrogenase was represented by the Hexa Uni Ping Pong mechanism, this enzyme showed no Sensitivity or Elasticity to NADH. However, when the more detailed mechanism for the enzyme, derived from the studies reported in Chapter IV section viii, was used, the enzyme showed a small amount of controllability. Under the conditions designated as 'normal', with floating NAD^+:NADH and acetylCoA:free CoA ratios, 2-oxoglutarate dehydrogenase shows a very small but positive Sensitivity. This may be taken to indicate that a situation could arise in which 2-oxoglutarate dehydrogenase becomes important to the regulation of Cycle flux, although it is impossible to predict under what conditions this may occur. Certainly there seems to be very little indication that variations in the NAD^+:NADH ratio mediated by 2-oxoglutarate dehydrogenase would have any effect on the Cycle.

The membrane bound enzyme succinic dehydrogenase also shows minor control properties under the conditions simulated. However it should be remembered that this enzyme has been simulated as being 'homogeneous' with the system, which is not the case in reality. No account has been taken in the simulations of the fact that succinate, in order to reach succinic

dehydrogenase, and fumarate, in order to reach fumarase, must pass across part of the inner mitochondrial membrane. Very little is known about the rates of diffusion of Citric Acid Cycle intermediates within the inner mitochondrial membrane. A preliminary study of how this situation could affect the operation of the Citric Acid Cycle has been carried out by Ottaway (1976). This study suggested that there may be some important kinetic effects on Cycle operation as a consequence of the position of succinic dehydrogenase within the membrane, particularly in the transition between two steady states. The fact that succinic-dehydrogenase is subject to a large number of allosteric controls (Singer et al., 1973) and appears to undergo a 'Activation - Deactivation' cycle (Ackrell et al., 1974) suggests that succinic dehydrogenase may play an important part in mitochondrial function, but we have already seen that such teleological arguments are not valid for NAD^+ linked isocitrate dehydrogenase in heart.

Another feature of succinic dehydrogenase which was not investigated in the simulation, and which may be very important, is the connection between it and the Electron Transport Chain. The reducing equivalents produced in the reaction are passed directly to the Electron Transport System at the level of cytochrome b. The activity of the enzyme is very possibly linked to the degree of reduction of the system accepting reducing equivalents from the flavoprotein. Succinic dehydrogenase would be an obvious site for co-ordination between the Citric Acid Cycle and electron transport, but at present it is impossible to investigate this as the actual electron acceptor for the enzyme is not known.

It has been shown that succinic dehydrogenase is not limiting for Cycle operation (Singer et al., 1973), but it has been suggested that the enzyme may function in the regulation of substrate levels in the mitochondrial matrix due to the possibility of the diffusion of fumarate through the membrane (see Ottaway, 1976) and in directing the flow through electron

transport (and hence oxidative phosphorylation) by virtue of its close contact with this system (Singer et al., 1973).

Succinyl thiokinase is a Cycle enzyme which has never previously been considered as a potential control point of the Citric Acid Cycle. Under most simulation conditions the enzyme in fact, showed no significant control properties. However, when the Cycle was supplied with acetoacetate, succinyl thiokinase showed very considerable negative Sensitivity. As explained in Section vii. of the previous Chapter this results from the dependence of the system on the concentration of succinyl CoA under these particular conditions. The phenomenon is interesting from two points of view. It serves as an illustration that an enzyme which shows very little or no control properties under most conditions can show considerable regulatory effects in certain situations. This particular case is especially relevant to heart tissue where ketones are a major source of oxidizable carbon. It had previously not been noted that this enzyme performs an important role in heart tissue under conditions of ketone metabolism, although the 'controllability' can to a great extent be removed by increasing the GTP:GDP ratio to values much higher than those used in the simulation studies (see Fig. IV.vii.). Since the actual GTP:GDP ratio within mitochondria is not known, it is impossible to say whether succinyl thiokinase is a controlling influence in vivo when acetoacetate is the carbon source.

That acetoacetate (and its reduced form β-hydroxy butyrate) is a major energy source in heart tissue is undisputed (Barnes et al., 1938; Hall, 1961; Williamson & Krebs, 1961; Olsen, 1962; Little et al., 1970), however the simulation studies indicate that the utilization of ketone bodies is not straightforward. The high K_m for succinylCoA of 3-keto acylCoA transferase implies that for this enzyme to operate efficiently a high concentration of succinylCoA must exist within the mitochondrion.

Under the usual simulation conditions the concentration of succinylCoA is not high enough to allow the pathway of conversion of acetoacetate to acetylCoA to operate, even at high concentrations of acetoacetate (1mmol/kg mito.). The most obvious way in which to increase the throughput of acetoacetate is to increase the steady state level of succinylCoA by retarding the succinyl thiokinase reaction. Altering the GTP:GDP.P_i ratio is perhaps the most plausible way of accomplishing this, both in the simulation studies and in the in vivo situation. This ratio has not been measured in heart tissue but experiments with perfused liver (Bryla et al., 1973) indicate that the GTP:GDP ratio is much higher than the ATP:ADP ratio, and that rapid equilibration of the adenine and guanine nucleotide pools through nucleoside diphosphate kinase cannot occur. It is possible that a similar situation exists in heart. It is encouraging that in the simulations where the ratio of GTP:GDP was high (section vii. Chapter IV) the conclusion that the start/finish junction of the Cycle is the major control point was upheld.

The existence of such a high ratio of GTP:GDP brings up the problem of how it can be brought about and maintained. If it is assumed that the ratios of GTP:GDP and ATP:ADP do differ markedly, the question then arises as to the stability of the relationship between the two. Will variations in the ATP:ADP ratio change the GTP:GDP ratio? Is the utilization of ketone bodies related to the phosphorylation state of not just the guanine nucleotides but also the adenine nucleotides? And conversely can the source of acetylCoA affect the ATP:ADP ratio?

Unfortunately as a result of the almost complete lack of information concerning these features of mitochondrial metabolism these questions must remain as unanswered. Not only would more information about the phosphorylation states of the adenine and guanine nucleotides be required but also more accurate details of kinetics, mechanism and intramitochondrial activities of the enzymes succinyl thiokinase, 3 keto acylCoA transferase

and nucleoside diphosphate kinase would be necessary before any firm conclusions about the utilization of ketone bodies in heart could be reached.

One feature of the study of the utilization of ketone bodies is that it provides a good illustration of the operation of a system becoming very sensitive to the concentration of one crucial metabolite, in this case succinylCoA. Even a slight sequestration of this metabolite has very great effects on the Citric Acid Cycle flux. Sequestration is of particular relevance to the mitochondrial pathways since many of the intermediates and co-enzymes are essentially 'trapped' within the mitochondrial matrix. The 'enclosedness' of the system means that substrate sequestration becomes an important feature and negative Sensitivities appear for some of the enzymes. This phenomenon indicates that increased binding of substrate to the enzyme(s) with which it is involved leads to a lowering of the free concentration of the metabolite and under certain conditions this can affect the flux through the Cycle. This feature was most evident with pyridine nucleotides. Even under 'normal' conditions the sequestration by the dehydrogenase enzymes, through the equilibrium reaction of malate dehydrogenase, elicits an alteration in the ratio of NAD^+:NADH such that Cycle flux is decreased (see Ottaway, 1976). During the oxidation of ketone bodies, the concentration of succinylCoA controls the flux through 3 keto acylCoA transferase, and hence the rate of production of acetylCoA. Any removal of succinylCoA from the system by sequestration decreases the rate of production of acetylCoA and Cycle flux falls in consequence.

Considering the number of Cycle intermediates and co-enzymes which cannot traverse the mitochondrial inner membrane, there is probably a number of situations other than those brought to light in these studies, where substrate sequestration becomes important to the flux through the Citric Acid Cycle. It is perhaps important, however, to re-iterate that sequestration is not important with respect to oxalacetate. Due to the

properties of the malate dehydrogenase reaction and the high intramitochondrial level of malate, sequestration of oxalacetate never becomes important to the functioning of the Cycle.

The conclusions reached in the simulation studies reported here to some extent agree with experimental studies and theoretical treatises published previously by other workers. The results agree with the views of Krebs & Lowenstein (1960) that, of the Cycle enzymes, citrate synthase is the most important controlling influence and furthermore the simulation studies show that all three factors suggested by these authors, namely the activity of citrate synthase itself, the concentration of oxalacetate and the concentration of acetylCoA are important to this regulation. The conclusions arrived at from the simulation studies also are in agreement with a later study by Krebs (1970) saying that the level of oxalacetate and the controls on the rate of production of acetylCoA are of major importance to Cycle flux. His finding that the level of ATP has great influence over the flux through citrate synthase could not be examined in these simulation studies since the electron transport system and oxidative phosphorylation were not included in the simulation.

There was no evidence whatsoever from these simulation studies to support the suggestion of Rolleston (1972) that citrate synthase is an 'equilibrium' enzyme. (The value of the Mass Action Ratio (Γ) for citrate synthase at steady state in the simulation is 0.7×10^{3}, and since the K_{eq} of the enzyme is 4.65×10^{5}, the value of Γ/K_{eq} is less than 0.02, the value proposed by Rolleston to be the upper limit for 'non-equilibrium' enzymes). Rolleston arrived at the conclusion that citrate synthase is an 'equilibrium' enzyme by evaluating Γ for citrate synthase from calculated intramitochondrial concentrations of oxalacetate, CoA, acetylCoA and citrate. These 'calculated' concentrations differ very markedly from those existing in the simulation, particularly with respect to the citrate concentration, which was calculated as being very much higher than malate in the mitochondrial matrix. These calculations were based on the assumption

that certain intramitochondrial and cytosolic enzymes are in equilibrium. Such a procedure is obviously open to error and it is most likely this is the basis for the discrepancy between Rolleston's finding and that of this study.

That citrate synthase is a major control point in the Cycle is in agreement with the findings of Randle, England and Denton (1970), and that the levels of oxalacetate and acetylCoA and the ratio of the pyridine nucleotides are all instrumental in this regulation agrees with the work of Williamson's group (LaNoue et al., 1970; 1972). However there was no evidence from the simulation studies to support the view of these latter authors (LaNoue et al., 1972) that succinylCoA can effectively inhibit citrate synthase, or the suggestion of Randle et al. (1970) that 2-oxoglutarate dehydrogenase is an important site of regulation of the Citric Acid Cycle.

The conclusions of Randle et al. (1970), were drawn from an examination of the changes in intramitochondrial substrate concentrations on changing from glucose to acetate metabolism. Although a change in the concentration of a particular intermediate may indicate a regulatory site (e.g. Crossover Theorem, Chance et al., 1958) this is not necessarily the case with the Citric Acid Cycle. In the Cycle there are three enzymes, the equilibria of which are dependent on the NAD^+:NADH ratio, yet all three need not indicate control characteristics if their equilibria change. This was quite evident from the studies reported in Chapter IV section i. When the NAD^+:NADH ratio is halved (Table IV.i.3) there is a significant drop (25%) in the concentration of 2-oxoglutarate, yet as the subsequent Elasticity studies show, 2-oxoglutarate dehydrogenase has a negligible Elasticity towards NADH i.e. the effect on Cycle flux elicited by NADH is not exerted through this enzyme. Examination of the variations in pool sizes alone do not constitute a reliable means of establishing control points of a system.

The unreliability of the measurement of changes in substrate concentrations as an indicator of 'regulatory' enzymes has been fully discussed by Rolleston (1972). He points out that the application of Crossover theorem (Chance et al., 1958) is limited to situations where there is conservation of intermediates, i.e., there is no means of removal of any intermediate other than by reaction with the enzymes catalysing conversion to the preceding or subsequent intermediate. This restriction in the application of the theorem has frequently been ignored and there are many cases in which results drawn from this Theorem are invalid (Rolleston, 1972).

Rolleston (1972) proposes that possible regulatory enzymes can be identified as those whose mass action ratio (Γ) is much less than the K_{eq} for the reaction, i.e. those reactions which are far from thermodynamic equilibrium. He suggests enzymic reactions where the value of Γ/K_{eq} is less than 0.05 be considered 'non-equilibrium' and those whose Γ/K_{eq} value is greater than 0.2 be considered as 'Equilibrium'. It is fundamental to his theory that 'Equilibrium' enzymes cannot be 'Regulatory' as his definition of 'Regulatory' states that such an enzyme must have an effect in the pathway flux by alterations in its activity. It is impossible for change in the activity of a 'Equilibrium' enzyme to affect pathway flux (Krebs, 1969; Hess, 1963; Bücher & Russman, 1964).

Rolleston (1972) also distinguishes between the terms 'Regulatory' enzyme and 'Rate-Limiting System', the latter being his descriptive term for all the factors composing the system of control. He quite rightly points out that regulation is not necessarily entirely due to the activity of one 'Regulatory' enzyme; the whole system of enzymes, cofactor ratios and substrate levels surrounding this enzyme are also involved in the control function. This view agrees very closely with the findings of this study with respect to the control 'system' operating at the start/finish junction of the Citric Acid Cycle.

Rolleston's strict definition of 'regulatory' is however misleading. As has been shown on the previous Chapter and discussed at length in this Chapter, malate dehydrogenase fulfils a very important regulatory role in the Citric Acid Cycle yet it falls into Rolleston's category of 'equilibrium' enzymes by virtue of having an Γ/K_{eq} value of 0.97 (Γ being calculated from values in Table IV.i.1), and thus cannot be considered as 'Regulatory' by Rolleston's standards.

It is this difficulty of definition which emphasizes the need for the application of a control theory such as that of Kacser & Burns (1973) or Heinrich & Rapaport, (1974) to simulation studies such as these. Such a procedure allows not only the identification of the controlling enzymes but also allows the control exerted to be quantified. This permits comparison of the relative importance of the various control points in the pathway to be made in a meaningful way. The procedure is also very useful in that the controlling effects of enzyme activity (Sensitivity) can be distinguished from the effects of changes in metabolite pool sizes (Elasticities). The calculation of both Sensitivity and Elasticity coefficients have been of fundamental importance to the analysis of the simulation studies. However, certain aspects of the theory of Kacser & Burns cannot be rigourously applied to the results. The theory states that all the Sensitivities in a system should sum up to a value of unity, that is where all the enzymes are increased by a factor of α, then the flux through the pathway should increase, also by a factor of α. This does not hold for the Citric Acid Cycle for a number of reasons:
i) the intermediates are unable to respond freely to changes in all the enzyme concentrations since the pathway is cyclic and oxalacetate is both substrate and product of the Cycle, thus increase in demand for substrate caused by raising the enzyme activities cannot be met entirely by factors outside the system itself. ii) the levels of the necessary co-enzymes for the pathway are fixed; these do not have the ability to increase in

concentration when the capacity of the enzymes for utilizing these is increased, iii) substrate sequestration by the enzymes is not negligible. Although these 'system limits' mean that the Summation Theorem cannot be applied, this does not rule out the application of the fundamental idea to this study, since absolute values of Sensitivity and Elasticity can be calculated from the simulation results. As discussed by Kacser & Burns (1973) it is only the application of the Summation Theorem which is restricted to a narrow range of sub-systems of metabolism where the effects listed previously are negligible.

Sensitivity and Elasticity coefficients are exceedingly difficult to measure experimentally in real systems, although much of the treatise of Kacser & Burns is concerned with the elucidation of the value from experimental measurement. Even so, in order to perform such calculations, there is a requirement for a great deal of information about the reactions and interrelationships within the system.

When the level of information about a system is great enough to allow a realistic simulation model to be constructed, such as in this study, the calculation of absolute Sensitivity and Elasticity coefficients from simulation results becomes very simple. There is the added advantage that with the very detailed output available from simulations using the CHEK program, the reasons underlying non-zero Sensitivity and Elasticity values can be examined, i.e., not only can the sites of control be identified, but the reasons for the regulatory action can be obtained. In this way it was possible to establish that the high Sensitivity of citrate synthase is due to the saturation of the enzyme by acetylCoA or that the effect of the NAD^+:NADH ratio on the Cycle is exerted almost entirely through malate dehydrogenase by virtue of the 'Equilibrium' nature of this reaction. The use of a simulation model to assess Sensitivity and Elasticity then has obvious advantages over attempts to arrive at these values from experimental studies or other theoretical treatments of the information

as described by Kacser & Burns (1973).

The majority of conclusions about the control of the Citric Acid Cycle reached in these simulation studies are not new. Most have been previously reached by experimental observation of teleological argument. However there was considerable confusion and argument concerning the relative physiological importance of the various proposed control points of the Cycle. Much of the confusion arose from the variety of tissues examined, of types of experiment (e.g. perfused tissue, isolated mitochondria) and of conditions of experiment (e.g. perfusion substrate). With such a mixture of facts and conclusions it was very difficult to see clearly how the Citric Acid Cycle is controlled in a physiological situation. Simulating the operation of the Cycle in one particular tissue, heart, and examining the control of the pathway under a series of different physiological conditions has greatly clarified the state of knowledge of how and when proposed control points become important and how they relate or interact with one another. It is very satisfying that the conclusions reached by simulation agree with many of those previously proposed from experimental studies, as this indicates both the validity of the simulation approach to the investigation of a biochemical system, and also the usefulness of combining experimental and simulation studies to reach more definite conclusions.

Although the importance of a number of proposed control points, such as isocitrate dehydrogenase, could be excluded by the simulation studies, it should be remembered that these need not be unimportant in other tissues or under conditions not examined in this study.

In any further extension of the work reported here it would be rewarding to study the control of the Cycle under a wider variety of conditions, i.e. where more of the ancillary pathways interacting with the Cycle are included. One particular area of mitochondrial metabolism whose

connection with the Citric Acid Cycle would be most important to study is the Electron Transport System. The fundamental importance of the NAD^+: NADH ratio to Cycle control has been clearly demonstrated and an extension of the simulation to include a fuller description of the Electron Transport System may give insight into how this ratio is itself regulated. Such an extended simulation may also be useful from the point of view of investigating the relationship between succinic dehydrogenase and the Electron Transport System.

. The rate of acetylCoA production has also been shown to be important in the control of Citric Acid Cycle flux and it would thus be useful to simulate the various routes of production of acetylCoA, such as β-oxidation and pyruvate dehydrogenase, in greater detail.

The simulation studies reported here refer exclusively to heart tissue. It is known however that the properties of the component enzymes of the Citric Acid Cycle vary from tissue to tissue. This may indicate that the Control of the Cycle differs from one tissue to another. This could be examined by simulating the Cycle according to the data for the various tissues. Although this would be a worthwhile project it would nevertheless involve rather a large amount of time, expense and energy.

The technique of simulation, of the type described in this thesis, has shown itself to be of great use on the analysis of a complex biochemical system. As a tool for biochemical research, simulation has many advantages. As has been demonstrated in the work reported here, it is very useful in confirming or discrediting proposals that have been arrived at previously from experimental observation, it may uncover anomalies which may not previously have been noted in experimental studies and it can indicate in which direction further experimental and simulation work would be most rewarding.

APPENDIX I

Examples of the subroutines INSET, COSTF, FINISH and FINAL used in the SIMPLEX optimizations.

Subroutine INSET used for SIMPLEX fitting of initial velocity data to rate equations for the 2-oxoglutarate dehydrogenase.

```
      SUBROUTINE INSET
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION ARRAY1(240),ARRAY2(240),ARRAY3(240),ARRAY(240)
      COMMON/CONST/ ARRAY1,ARRAY2,ARRAY3,ARRAY
      M=-1.0
      DO 103 N=1,60
      READ(5,101)A,B,C
101   FORMAT(1H0,3F6.4)
      M=M+2
      J=M+1
      DO 102 I=M,J
      ARRAY1(I)=A
      ARRAY2(I)=B
      ARRAY3(I)=C
      READ(5,104)ARRAY(I)
104   FORMAT(1H ,F7.6)
      IF(ARRAY(I).EQ.0.00000) GOTO90
      GOTO 102
90    ARRAY1(I)=0.0000
      ARRAY2(I)=0.0000
      ARRAY3(I)=0.0000
102   CONTINUE
103   CONTINUE
      RETURN
      END
```

COSTF subroutine used for SIMPLEX fitting of initial velocity data to mechanism 4 (Fig. IV.vii.7) for 2-oxoglutarate dehydrogenase.

```
      SUBROUTINE COSTF(X,N,COST)
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION X(N),DEV(240),V(240),DIFF(240),PCENT(240),ARR(240)
      COMMON/CONST/ ARRAY1(240),ARRAY2(240),ARRAY3(240),ARRAY(240),DEV,
     1V,PCENT,C1,C2,C3,C4,C5,C6,C7,C8,C9,C10,C11,C12,C13,C14,SSQ
C     THIS IS AN ATTEMPT TO FIT THE EXPERIMENTAL DATA DIRECTLY
C     TO THE INITIAL RATE EQUATION FOR THE 1ST SEMI RANDOM MECHANISM
C     DEVS ARE CALC'D AS PROPORTIONAL DEVS-I E-(V CALC.V)/V+CALC.V
C     MECHANISM STUDIES   17.12.75
      DO 100 J=1,N
      IF(X(J).GE.1.0E8) X(J)=1.0E8
  100 IF(X(J).LE.1.0E-8) X(J)=1.0E-8
      C1=X(1)
      C2=X(2)
      C3=X(3)
      C4=X(4)
      C5=X(5)
      C6=X(6)
      C7=X(7)
      C8=X(8)
      C9=X(9)
      C10=X(10)
      C11=X(11)
      C12=X(12)
      C13=X(13)
      C14=X(14)
      COST=0.0D0
      DO 101 I=1,120
      IF(ARRAY1(I).EQ.0.0000)GOTO102
      V(I)=(C1*ARRAY1(I)*ARRAY2(I)*ARRAY3(I)+C2*ARRAY1(I)*ARRAY2(I)
     1*ARRAY2(I)*ARRAY3(I)+C3*ARRAY1(I)*ARRAY2(I)*ARRAY3(I)*ARRAY3(I))
     2/(C4*ARRAY1(I)+C5*ARRAY1(I)*ARRAY2(I)+C6*ARRAY1(I)*ARRAY3(I)
     3+C7*ARRAY1(I)*ARRAY2(I)*ARRAY2(I)+C8*ARRAY1(I)*ARRAY3(I)*ARRAY3
     4(I)+C9*ARRAY2(I)*ARRAY3(I)+C10*ARRAY1(I)*ARRAY2(I)*ARRAY3(I)+C11
     5*ARRAY1(I)*ARRAY2(I)*ARRAY3(I)*ARRAY3(I)+C12*ARRAY1(I)*ARRAY2(I)
     6*ARRAY2(I)*ARRAY3(I)+C13*ARRAY2(I)*ARRAY3(I)*ARRAY3(I)+C14*
     7ARRAY2(I)*ARRAY2(I)*ARRAY3(I))
      ARR(I)=ARRAY(I)/60.0
      DIFF(I)=(ARR(I)-V(I))/(ARR(I)+V(I))
      DEV(I)=DIFF(I)*DIFF(I)
      PCENT(I)=((ARR(I)-V(I))/ARR(I))*100.0
      COST=COST+DEV(I)
      SSQ=COST
      GOTO 101
  102 V(I)=0.0
      DEV(I)=0.0
      PCENT(I)=0.0
  101 CONTINUE
      RETURN
      END
```

Subroutines FINISH and FINAL for SIMPLEX fitting of initial velocity data to mechanism 4 (Fig. IV.vii.7) for 2-oxoglutarate dehydrogenase.

```
      SUBROUTINE FINISH
      IMPLICIT REAL*8(A-H,O-Z)
      COMMON/CONST/ ARRAY1(240),ARRAY2(240),ARRAY3(240),ARRAY(240),
     1DEVV(240),V(240),PCENT(240),C1,C2,C3,C4,C5,C6,C7,C8,C9,C10,C11,
     2C12,C13,C14,COST
      WRITE(6,201)
201   FORMAT(1H0,'    C1          C2          C3          C4          C5
     1     C6          C7     ')
      WRITE(6,202)C1,C2,C3,C4,C5,C6,C7
202   FORMAT(1H0,6(F8.3,1X,)F8.3,/)
      WRITE(6,203)
203   FORMAT(1H0,'    C8          C9          C10         C11         C12         C13
     1       C14    ')
      WRITE(6,204)C8,C9,C10,C11,C12,C13,C14
204   FORMAT(1H0,6(F8.3,1X,)F8.3)
      WRITE(6,200)COST
 200  FORMAT(//,'THE COST IS =',E15.7)
      RETURN
      END

      SUBROUTINE FINAL
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION RECIP(240),REC(240)
      COMMON/CONST/ ARRAY1(240),ARRAY2(240),ARRAY3(240),ARRAY(240),
     1DEV(240),V(240),PCENT(240)
      WRITE(6,206)
 206  FORMAT(1H0,'  K   CONC.A',2X,'CONC.B',2X,'CONC.C',3X,'INT.V',5X,
     1'CALC.V',7X,'DEV',6X,'%',8X,'1/INT.V',7X,'1/CALC.V',//)
      DO 207 K=1,120
      IF(ARRAY(K).EQ.0.0000)GOTO111
      IF(V(K).EQ.0.0000)GOTO112
      RECIP(K)=1.0/ARRAY(K)
      REC(K)=1.0/(V(K)*60.0)
      GOTO113
 111  RECIP(K)=0.0
 112  REC(K)=0.0
 113  WRITE(6,205)K,ARRAY1(K),ARRAY2(K),ARRAY3(K),ARRAY(K),V(K),DEV(K)
     1,PCENT(K),RECIP(K),REC(K)
 205  FORMAT(1H0,I3,1X,F6.4,2X,F6.4,2X,F6.4,2X,F8.6,2X,F10.8,2X,E10.5,
     12X,F6.2,2X,E12.5,2X,E12.5)
 207  CONTINUE
      RETURN
      END
```

COSTF subroutine used to fit rate constants to the Hexa Uni Ping Pong mechanism for 2-oxoglutarate dehydrogenase.

```
      SUBROUTINE COSTF(X,N,COST)
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION X(N)
      COMMON/CONST/ VMAX, EQK, AKM, COKM, DKM, SKM
      DO 110 J=1,N
      IF(X(J).LT.1.0D-6) X(J)=1.0D-6
  110 IF(X(J).GT.1.0D8) X(J)=1.0D8
      C1=X(1)
      C2=X(2)
      C3=X(3)
      C4=X(4)
      C5=X(5)
      C6=X(6)
      C7=X(7)
      C8=X(8)
      C9=X(9)
      C10=X(10)
      COST=0.0D0
C     THIS IS THE VMAX FOR THE FORWARD REACTION
      VMAX=(C3*C7)/(C3+C7)
      DEV=(570.0-VMAX)/10.0
      DEV=DEV*DEV
      COST=COST+DEV
C      THIS IS THE KM FOR OXOGLUTARATE
      AKM=(C3*C7)/(C1*(C3+C7))
      DEV=(0.013-AKM)*10000.0
      DEV=DEV*DEV
      COST=COST+DEV
C      THIS IS THE KM FOR COA
      COKM=(C7*(C3+C4))/(C5*(C3+C7))
      DEV=(0.0001-COKM)*100000.0
      DEV=DEV*DEV
      COST=COST+DEV
C      THIS IS THE KM FOR NAD
      DKM=(C3*(C7+C8))/(C9*(C3+C7))
      DEV=(0.0045-DKM)*10000.0
      DEV=DEV*DEV
      COST=COST+DEV
C      THIS IS THE KM FOR SUCCINYLCOA
      SKM=(C4*(C7+C8))/(C6*(C4+C8))
      DEV=(0.03-SKM)*1000.0
      DEV=DEV*DEV
      COST=COST+DEV
C      THIS IS THE EQUILIBRIUM CONSTANT
      EQK=(C1*C3*C5*C7*C9)/(C2*C4*C6*C8*C10)
      DEV=(7.5D5-EQK)/10000.0
      DEV=DEV*DEV
      COST=COST+DEV
      RETURN
      END
```

Subroutines FINISH, FINAL and INSET used in the fitting of rate constants for the Hexa Uni Ping Pong mechanism for 2-oxoglutarate dehydrogenase.

```
      SUBROUTINE FINISH
      IMPLICIT REAL*8(A-H,O-Z)
      COMMON/CONST/ VMAX, EQK, AKM, COKM, DKM, SKM
      WRITE(6,91)
91    FORMAT(1H0,'  VMAX       EQK       DKM       AKM       COKM       SKM'.
     *' 570.0    7.5D5    0.0045    0.013   0.0001     0.03')
      WRITE(6,92) VMAX, EQK, DKM, AKM, COKM, SKM
92    FORMAT(//,F8.2,D8.1,F8.4,F8.4,F8.5,F8.4)
      RETURN
      END

      SUBROUTINE INSET
      RETURN
      END

      SUBROUTINE FINAL
      RETURN
      END
```

COSTF subroutine used for the fitting of rate constants to the mechanism for isocitrate dehydrogenase.

```
      SUBROUTINE COSTF(X,N,COST)
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION X(N)
      COMMON/CONST/ VMAXF,EQK,XKMNAD,XKMIC,XKNADH,XKICO2,XKIAOG
      DO 110 J=1,N
      IF(X(J).LT.1.0D-5) X(J)=1.0D-5
  110 IF(X(J).GT.1.0D6) X(J)=1.0D6
      C1=X(1)
      C2=X(2)
      C3=X(3)
      C4=X(4)
      C5=X(5)
      C6=X(6)
      C7=X(7)
      C8=X(8)
      C9=X(9)
      C10=X(10)
      COST=0.0D0
C     THIS IS THE VMAX FOR THE FORWARD REACTION
      VMAXF=(C5*C7*C9)/(C5*C7+C7*C9+C5*C9)
      DEV=(133.0-VMAXF)
      DEV=DEV*DEV
      COST=COST+DEV
C     THIS IS THE KM FOR NAD
      XKMNAD=(C5*C7*C9)/(C1*(C5*C7+C7*C9+C5*C9))
      DEV=(0.08-XKMNAD)*100.0
      DEV=DEV*DEV
      COST=COST+DEV
C     THIS IS THE KM FOR ISOCITRATE
      XKMIC=(C7*C9*(C4+C5))/(C3*(C5*C7+C7*C9+C5*C9)
      DEV=(0.14-XKMIC)*100.0
      DEV=DEV*DEV
      COST=COST+DEV
C      THIS IS THE KI FOR NADH
      XKNADH=C9/C10
      DEV=(0.04-XKNADH)*100.0
      DEV=DEV*DEV
      COST=COST+DEV
C     THIS IS THE EQUILIBRIUM CONSTANT
      EQK=(C1*C3*C5*C7*C9)/(C2*C4*C6*C8*C10)
      DEV=(855.0-EQK)
      DEV=DEV*DEV
      COST=COST+DEV
C     THESE ARE THE DUMMY VALUES OF KI FOR CO2 AND OXOGLUTARATE
      XKICO2=C5/C6
      DEV=(1.0E3-XKICO2)/(1.0E3+XKICO2)
      DEV=DEV*DEV
      COST=COST+DEV
      XKIAOG=C7/C8
      DEV=(1.0E3-XKIAOG)/(1.0E3+XKIAOG)
      DEV=DEV*DEV
      COST=COST+DEV
      RETURN
      END
```

APPENDIX II

Examples of the input files used for CHEK simulations.

******** THIS IS THE INPUT FILE ********

```
* ; DEMONSTRATION OF ENZYME SIMULATOR PROGRAM
* ; SIMULATION OF  FUMARASE AND MALATE DEHYDROGENASE
* ; FEBRUARY 1976    USING CHEK VERSION OF PROGRAM
** ;
```

----- COMMENTS

```
  CONCENTRATIONS          RATE CONSTANTS
    /       /               /       /
   /       /               /       /
  /       /               /       /
0.001 E + 1.0 FUM = EFUM  / 0.11E+8 / 0.27E+5  ;
EFUM = EMAL / 0.23E+4 / 0.17E+4  ;
EMAL = E + MAL /  0.46E+5 / 0.5E+7  ;
0.002 MDH + 1.0 NAD = MDHNAD  /  5.5E2  /  5.7E2  ;
MDHNAD + MAL = MDHXY   /  2.0E3  /  3.3E4  ;
MDHXY = MDHNADH + OAA   /  8.3E3  /  1.7E4  ;
MDHNADH = MDH + NADH   /  1.7E2  /  3.3E4  ;
NADH = NAD   /  10.  ;
** ;
```

----- EQUATION DECK

```
MAX -0.001 E EFUM EMAL  ;
MAX -1.0 FUM MAL NAD NADH OAA  ;
MAX -0.001 MDH MDHNAD MDHXY MDHNADH  ;
GRAPH OAA 0.5 0  ;
GRAPH FUM 1.0 F  ;
GRAPH MAL 1.0 M  ;
NSTEP  40  ;
DELTA  0.05  ;
BEGIN  ;
LIST  2  ;
NSTEP  50  ;
DELTA  0.1  ;
RUN  ;
LIST  2  ;
STOP  ;
```

----- COMMAND DECK

```
* ; SIMULATION OF THE T.C.A. CYCLE 21/10/74
* ; STEADY STATE SIMULATION
** ;
0.01 ST + 0.22E-2 SUCOA = STSUCOA / 370. / 11.4 ;
STSUCOA + 0.7211 P = ST*SUP / 43.8 / 65.2 ;
ST*SUP = ST*P + SUCC / 66.7 / 24.0 ;
ST*P + 0.9786E-1 GDP = ST*GTP / 2268. / 27.2 ;
ST*GTP = ST* + 0.1504E-2 GTP / 1217. / 40804. ;
ST* = ST + COA / 13.4 / 547.4 ;
0.23E-2 SDH + 0.289E-1 SUCC = SDHSUCC / 36. / 2.2 ;
SDHSUCC + 0.5 AOX = SDHFUM + 0.5 ARED / 7.7 ;
SDHFUM = SDH + FUM / 130. / 69. ;
0.8E-2 FASE + 0.986E-1 FUM = FASEFUM / 0.11E8 / 0.27E5 ;
FASEFUM = FASEMAL / 0.23E4 / 0.17E4 ;
FASEMAL = FASE + MAL / 0.46E5 / 0.5E7 ;
0.07 MDH + 0.2372 NAD = MDHNAD / 5.5E2 / 5.7E2 ;
MDHNAD + 0.5 MAL = MDHXY / 2.0E3 / 3.3E4 ;
MDHXY = MDHNADH + OAA / 8.3E3 / 1.7E4 ;
MDHNADH = MDH + 0.2674 NADH / 1.7E2 / 3.3E4 ;
1.0 PREC1 = 1.0 PREC1 + 0.33E-1 ACCOA / 33. / 1E3 ;
0.026 CS + 0.6342E-4 OAA = CSOAA / 1E3 / 5. ;
CS + ACCOA = CSACCOA / 1E3 / 4.5 ;
CS + SUCOA = CSI / 1E3 / 130. ;
CSOAA + ACCOA = CSOAAACCOA / 1E3 / 4.5 ;
CSACCOA + OAA = CSOAAACCOA / 1E3 / 5. ;
CSOAA + SUCOA = CSI* / 1E3 / 130. ;
CSOAAACCOA = CSCITCOA / 283. / 0.2 ;
CSCITCOA = CSCOA + CIT / 300. / 1E3 ;
CSCITCOA = CSCIT + COA / 30. / 1E3 ;
CSCOA = CS + COA / 30. / 1E3 ;
CSCIT = CS + CIT / 300. / 1E3 ;
0.074 AC + 0.2089E-1 CIT = ACCIT / 550. / 1330. ;
ACCIT = ACX / 95. / 65. ;
ACX = ACA / 1210. / 9200. ;
ACA = AC + A / 360. / 750. ;
ACX = ACIC / 2900. / 2300. ;
ACIC = AC + IC / 43. / 510. ;
0.092 ID + NAD = IDNAD / 1755. / 4874. ;
IDNAD + 0.1278E-2 IC = IDX / 1396. / 476. ;
IDX = IDY + CO2 / 1181. / 3.8 ;
IDY = IDNADH + AOG / 1660. / 26. ;
IDNADH = ID + NADH / 165. / 4082. ;
ID + ATP = IDI / 1E3 / 150. ;
0.006 KGDH1 + 0.8011E-5 AOG = KGDH2 + CO2 / 4.4E4 / 1.24E5 ;
KGDH2 = KGDH3 / 1.73E4 / 66.0 ;
KGDH3 + COA = KGDH4 + SUCOA / 5.7E6 / 1.12E4 ;
KGDH4 = KGDH5 / 590. / 62. ;
KGDH5 + NAD = KGDH1 + NADH / 1.4E5 / 8.35E4 ;
CO2 = BICARB / 100. ;
NADH = NAD / 4700. / 5300. ;
0.711E-1 ATP = 4.633 ADP + P / 470. / 10. ;
ADP + GTP = ATP + GDP / 100. / 100. ;
AOX = ARED / 100. / 100. ;
1.0 REM + 0.066 COA = 1.0 REM / 1000. / 66. ;
** ;
```

```
MAX -0.2234E-2 SUCOA  ;
MAX -0.9786E-1 GDP  ;
MAX -0.1504E-2 GTP  ;
MAX -0.289E-1 SUCC  ;
MAX -0.5 AOX ARED  ;
MAX -0.986E-1 FUM  ;
MAX -0.5 MAL  ;
MAX -0.2372 NAD  ;
MAX -0.2674 NADH  ;
MAX -0.033 ACCOA  ;
MAX -0.6342E-4 OAA  ;
MAX -0.2089E-1  CIT  ;
MAX -0.1278E-2 IC  ;
MAX -0.8071E-5 AOG  ;
MAX -0.066 COA  ;
MAX -0.01 ST STSUCOA ST*SUP ST*P ST*GTP ST*  ;
MAX -0.23E-2 SDH SDHSUCC SDHFUM  ;
MAX -0.8E-2 FASE FASEFUM FASEMAL  ;
MAX -0.07 MDH MDHNADH MDHNAD MDHXY  ;
MAX -1.0 PREC1 REM  ;
MAX -0.026 CS CSOAA CSOAAACCOA CSACCOA CSI CSCITCOA CSI*  ;
MAX -0.026 CSCIT CSCOA  ;
MAX -0.074 AC ACCIT ACX ACA ACIC  ;
MAX -0.001 A  ;
MAX -0.092 ID IDNAD IDX IDY IDNADH IDI  ;
MAX -0.006 KGDH1 KGDH2 KGDH3 KGDH4 KGDH5  ;
MAX -0.711E-1 ATP  ;
MAX -4.633 ADP  ;
MAX -0.1E-3 CO2 BICARB  ;
GRAPH CIT 0.3 C  ;
GRAPH MAL 0.7 M  ;
GRAPH OAA 0.0001  O  ;
GRAPH IC 0.005  I  ;
GRAPH  AOG 0.5E-4  A  ;
GRAPH SUCOA 0.01  S  ;
NSTEP 50  ;
DELTA 3.0  ;
BEGIN  ;
LIST  2  ;
NSTEP 50  ;
DELTA 3.0  ;
RUN  ;
LIST  2  ;
NSTEP 50  ;
DELTA 3.0  ;
RUN  ;
LIST 2  ;
NSTEP  50  ;
DELTA  3.0  ;
RUN  ;
LIST  2  ;
NSTEP  50  ;
DELTA 3.0  ;
RUN  ;
LIST  2  ;
STOP  ;
```

APPENDIX III

Example of the output obtained from a CHEK simulation.

```
***ERCC 4-75 EMAS*** EQBG01   AITCHISON   MCMINN:ALISON.HO   21/10/76   15.23.00

      EXAMPLE  OF  OUTPUT FROM  THE  CHEK  SIMULATION  PROGRAM


          /          * ; SIMULATION OF THE T.C.A. CYCLE 21/10/74
-                    * ; STEADY STATE SIMULATION
                     ** ;

 ************************************ EQUATION SOURCE DECK ********************************
EQN.
  NO.    REAC. NO.                          EQUATION

                     0.01 ST + 0.22E-2 SUCOA =  STSUCOA  /  370.  /  11.4   ;
+   1       1    2

                     STSUCOA + 0.7211 P = ST*SUP  /  43.8  /  65.2  ;
+   2       3    4

                     ST*SUP =  ST*P +  SUCC  /  66.7  /  24.0   ;
+   3       5    6

                     ST*P + 0.9706E-1 GDP =  ST*GTP  /  2269.  /  27.2  ;
+   4       7    8

                     ST*GTP = ST* + 0.1504E-2 GTP  /  1217.  /  40894.  ;
+   5       9   10

                     ST* = ST + COA  /  13.4  /  547.4   ;
+   6      11   12

                     0.23E-2 SDH + 0.289E-1 SUCC = SDHSUCC  /  36.  /  2.2  ;
+   7      13   14
```

```
                  SDHSUCC + 0.5 AOX = SDHFUM + 0.5 ARED  /  7.7   ;
+   8       15

                  SDHFUM = SDH + FUM  /  130.  /  69.    ;
+   9     16,  17

                  0.8E-2 FASE + 0.986E-1 FUM =  FASEFUM  /  0.11E8  /  0.27E5  ;
+  10     18   19

                  FASEFUM = FASEMAL  / 0.23E4  /  0.17E4   ;
+  11     20   21

                  FASEMAL = FASE + MAL  /  0.46E5  /  0.5E7   ;
+  12     22   23

                  0.07 MDH + 0.2372 NAD =  MDHNAD  /  5.5E2  /  5.7E2  ;
+  13     24   25

                  MDHNAD + 0.5 MAL =  MDHXY  /  2.0E3  /  3.3E4  ;
+  14     26   27

                  MDHXY = MDHNADH + OAA  /  8.3E3  /  1.7E4   ;
+  15     28   29

                  MDHNADH = MDH + 0.2674 NADH  /  1.7E2  /  3.3E4  ;
+  16     30   31

                  1.0 PREC1 = 1.0 PREC1 + 0.33E-1 ACCOA  /  33.  /  1E3   ;
+  17     32   33

                  0.026 CS + 0.6342E-4 OAA =  CSOAA  /  1E3  /  5.  ;
+  18     34   35
```

```
+  19     36   37    CS + ACCOA =  CSACCOA  /  1E3  /  4.5  ;

+  20     38   39    CS + SUCOA =  CSI  /  1E3  /  130.  ;

+  21     40   41    CSOAA + ACCOA = CSOAAACCOA  /  1E3  /  4.5  ;

+  22     42   43    CSACCOA + OAA = CSOAAACCOA  /  1E3  /  5.  ;

+  23     44   45    CSOAA + SUCOA =  CSI*  /  1E3  /  130.  ;

+  24     46   47    CSOAAACCOA  = CSCITCOA  /  283.  /  0.2  ;

+  25     48   49    CSCITCOA  = CSCOA + CIT  /  300.  /  1E3  ;

+  26     50   51    CSCITCOA = CSCIT + COA  /  30.  /  1E3  ;

+  27     52   53    CSCOA = CS + COA  /  30.  /  1E3  ;

+  28     54   55    CSCIT = CS + CIT  /  300.  /  1E3  ;

+  29     56   57    0.074 AC + 0.2889E-1 CIT = ACCIT  /  550.  /  1330.  ;
```

```
                                ACCIT = ACX /  95.  /  65.  ;
+  30      58   59
                                ACX = ACA  /  1210.  /  9200.  ;
+  31      60   61
                                ACA = AC +  A  /  360.  /  730.  ;
+  32      62   63
                                ACX = ACIC  /  2900.  /  2300.  ;
+  33      64   65
                                ACIC = AC + IC  /  43.  /  510.  ;
+  34      66   67
                                0.092 ID + NAD = IDNAD  /  1755.  /  4874.  ;
+  35      68   69
                                IDNAD + 0.1278E-2 IC =  IDX  /  1396.  /  476.  ;
+  36      70   71
                                IDX = IDY + CO2  /  1181.  /  3.8  ;
+  37      72   73
                                IDY = IDNADH + AOG  /  1660.  /  26.  ;
+  38      74   75
                                IDNADH = ID + NADH  /  165.  /  4082.  ;
+  39      76   77
                                ID + ATP = IDI  /  1E3  /  150.  ;
+  40      78   79
```

```
                                  0.006  KGDH1 + 0.8011E-5 AOG = KGDH2 + CO2   / 4.4E4  / 1.24E5  ;
+  41      80   81
                                  KGDH2 = KGDH3  / 1.73E4  / 66.0   ;
+  42      82   83
                                  KGDH3 + COA = KGDH4 + SUCOA   / 5.7E6  / 1.12E4   ;
+  43      84   85
                                  KGDH4 = KGDH5   / 590.  / 62.    ;
+  44      86   87
                                  KGDH5 + NAD = KGDH1  +  NADH   / 1.4E5  / 8.35E4  ;
+  45      88   89
                                  CO2 = BICARB  / 100.  ;
+  46          90
                                  NADH = NAD   / 4700.  / 5300.    ;
+  47      91   92
                                  0.711E-1 ATP = 4.633 ADP + P  / 470.  / 10.  ;
+  48      93   94
                                  ADP + GTP = ATP + GDP  / 100.  / 100.  ;
+  49      95   96
                                  AOX = ARED  / 100.  / 100.  ;
+  50      97   98
                                  1.0 REN + 0.066 COA = 1.0 REN   / 1000.  / 66.   ;
+  51      99  100
```

```
                        ** ;
*****END OF EQUATION LIST
NO. OF EQUATIONS COMPILED =          51
NO. OF REACTIONS COMPILED =         100
NO. OF VARIABLES COMPILED =          65
NO. OF ERRORS =                       0
NO. OF DIFFERENT RATE CONSTANTS =   100
NO. OF OCCURRENCES =                199

COMMAND INTERPRETER ENTERED
          MAX -0.2234E-2 SUCOA ;
          MAX -0.9786E-1 GDP  ;
          MAX -0.1584E-2 GTP  ;
          MAX -0.289E-1 SUCC  ;
          MAX -0.5 AOX ARED  ;
          MAX -0.986E-1 FUM  ;
          MAX -0.5 MAL  ;
          MAX -0.2372 NAD  ;
          MAX -0.2674 NADH  ;
          MAX -0.033 ACCOA  ;
          MAX -0.6342E-4 OAA  ;
          MAX -0.2889E-1  CIT  ;
          MAX -0.1278E-2 IC  ;
          MAX -0.8871E-5 AOG  ;
          MAX -0.866 COA  ;
```

```
          MAX -0.01 ST STSUCOA ST*SUP ST*P ST*GTP ST*  ;
          MAX -0.23E-2 SDH SDHSUCC SDHFUM  ;
          MAX -0.8E-2 FASE FASEFUM FASEMAL  ;
          MAX -0.07 MDH MDHNADH MDHNAD MDHXY  ;
          MAX -1.0 PREC1 REM  ;
          MAX -0.026 CS CSOAA CSOAAACCOA CSACCOA CSI CSCITCOA CSI*  ;
          MAX -0.026 CSCIT CSCOA  ;
          MAX -0.074 AC ACCIT ACX ACA ACIC  ;
          MAX -0.001 A  ;
          MAX -0.092 ID IDNAD IDX IDY IDNADH IDI  ;
          MAX -0.006 KGDH1 KGDH2 KGDH3 KGDH4 KGDH5  ;
          MAX -0.711E-1 ATP  ;
          MAX -4.633 ADP  ;
          MAX -0.1E-3 CO2 BICARB  ;
          GSTREAM 6 70  ;
          GRAPH CIT 0.3 C  ;
          GRAPH MAL 0.7 M  ;
          GRAPH OAA 0.0001  O  ;
          GRAPH IC 0.005  I  ;
          GRAPH  AOG 0.5E-4  A  ;
          GRAPH SUCOA 0.01  S  ;
          NSTEP  10  ;
          DELTA  5.0  ;
          BEGIN  ;
MATRIX LOCATIONS NEEDED
FOR B AND IRN        339
FOR ICP               14
FOR A AND IRNA       540
```

```
PLOTTING PARAMETERS
CIT                    0.3000E 00 C
MAL                    0.7000E 00 M
OAA                    0.1000E-03 O
IC                     0.5000E-02 I
AOG                    0.5000E-04 A
SUCOA                  0.1000E-01 S
 0.0000E 1 .----C----'A---S--I-'---------'---------'---O-----M---------'---------.
 0.5000E 1 .    C    A'   S  I   '         '         '   O     M'         '         .
 1.0000E 1 .    C     A  S  I    '         '         '   O     M'         '         .
 1.5000E 1 .     C    A   S  I   '         '         '   O     M'         '         .
 2.0000E 1 .     C    A   S  I   '         '         '   O     M'         '         .
 2.5000E 1 .     C    A   S   I  '         '         '   O     M'         '         .
 3.0000E 1 .     C    'A   S  I  '         '         '   O     M'         '         .
 3.5000E 1 .     C    'A   S  I  '         '         '   O     M'         '         .
 4.0000E 1 .     C    'A   S  I  '         '         '   O     M'         '         .
 4.5000E 1 .     C    'A   S  I  '         '         '   O     M'         '         .
 5.0000E 1 .----C----'A---S--I-'---------'---------'---O----M'---------'---------.

COMMAND INTERPRETER ENTERED
          NSTEP  70   ;
          DELTA  10.0  ;
          RUN   ;

 0.6000E 2 .     C    'A   S   I'         '         '   O     M'         '         .
 0.7000E 2 .     C    'A   S   I'         '         '   O     M'         '         .
 0.8000E 2 .     C    'A   S   I'         '         '   O     M'         '         .
 0.9000E 2 .     C    'A   S   I'         '         '   O     M'         '         .
 1.0000E 2 .     C    'A   S   I'         '         '  O      M'         '         .
 1.1000E 2 .     C    'A   S   I'         '         '  O      M'         '         .
 1.2000E 2 .     C    'A   S   I'         '         '  O      M'         '         .
 1.3000E 2 .     C    'A   S   I'         '         '  O      M'         '         .
 1.4000E 2 .     C    'A   S   I'         '         '  O      M'         '         .
 1.5000E 2 .----C----'A---S---I'---------'---------'--O-----M'---------'---------.
```

```
1.6000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
1.7000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
1.8000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
1.9000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
2.0000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
2.1000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
2.2000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
2.3000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
2.4000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
2.5000E 2 .-----C-----'A---S---I'----------'----------'--0------M'----------'----------.
2.6000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
2.7000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
2.8000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
2.9000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
3.0000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
3.1000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
3.2000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
3.3000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
3.4000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
3.5000E 2 .-----C-----'A---S---I'----------'----------'--0------M'----------'----------.
3.6000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
3.7000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
3.8000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
3.9000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
4.0000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
4.1000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
4.2000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
4.3000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
4.4000E 2 .     C     'A    S    I'          '          '  0      M'          '          .
4.4999E 2 .-----C-----'A---S---I'----------'----------'--0------M'----------'----------.
```

```
 4.5999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 4.6999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 4.7999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 4.8999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 4.9999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 5.0999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 5.1999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 5.2999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 5.3999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 5.4999E 2 .-----C-----'A---S---I'----------'----------'--0------M'----------'----------.
 5.5999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 5.6999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 5.7999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 5.8999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 5.9999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 6.0999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 6.1999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 6.2999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 6.3999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 6.4999E 2 .-----C-----'A---S---I'----------'----------'--0------M'----------'----------.
 6.5999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 6.6999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 6.7999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 6.8999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 6.9999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 7.0999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 7.1999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 7.2999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 7.3999E 2 .     C     'A    S    I'          '          '  0      M'          '          .
 7.4999E 2 .-----C-----'A---S---I'----------'----------'--0------M'----------'----------.
COMMAND INTERPRETER ENTERED
          LIST  2  ;
```

*****CONCENTRATION AND REACTION FLUX TABLE

- STARTING VALUES

INDEX	NAME OF VARIABLE	CONC.	DERIV.	MAX VALUE
1	ST	2.5485D-03	-7.1774D-17	-1.0000E-02
2	SUCOA	2.1957D-03	-5.5663D-16	-2.2340E-03
3	STSUCOA	7.6622D-05	-1.8431D-17	-1.0000E-02
4	P	7.1463D-01	4.7566D-14	7.2110E-01
5	ST*SUP	1.8426D-05	-2.7165D-18	-1.0000E-02
6	ST*P	4.8165D-05	-4.2284D-18	-1.0000E-02
7	SUCC	2.7742D-02	3.5230D-14	-2.8900E-02
8	GDP	9.7531D-02	-1.8041D-16	9.7531E-02
9	ST*GTP	3.4769D-04	1.8041D-16	-1.0000E-02
10	ST*	6.9606D-03	-8.3267D-17	-1.0000E-02
11	GTP	1.4856D-03	0.0000D 00	-1.5040E-03
12	COA	6.6001D-02	-2.5596D-14	6.6001E-02
13	SDH	1.8834D-03	-3.1919D-16	-2.3000E-03
14	SDHSUCC	3.1090D-04	3.4364D-16	-2.3000E-03
15	AOX	4.9999D-01	1.4244D-15	-5.0000E-01
16	SDHFUM	1.0572D-04	-2.4449D-17	-2.3000E-03
17	ARED	5.0001D-01	-1.4244D-15	5.0001E-01
18	FUM	9.6551D-02	1.6364D-14	-9.8600E-02
19	FASE	8.5508D-05	1.4211D-14	-8.0000E-03
20	FASEFUM	3.3635D-03	-1.9318D-14	-8.0000E-03
21	FASEMAL	4.5499D-03	5.1078D-15	-8.0000E-03
22	MAL	4.8953D-01	-3.0642D-14	-5.0000E-01
23	MDH	1.8039D-03	1.3461D-15	-7.0000E-02
24	NAD	1.7191D-01	-3.5393D-13	1.7191E-01
25	MDHNAD	2.9713D-04	-1.3878D-17	-7.0000E-02
26	MDHXY	8.7791D-06	-1.3878D-17	-7.0000E-02
27	MDHNADH	6.7890D-02	-1.3184D-15	-7.0000E-02
28	OAA	6.2098D-05	-8.6736D-19	6.2098E-05
29	NADH	1.9386D-01	3.5527D-13	-2.6740E-01
30	PREC1	1.0000D 00	0.0000D 00	-1.0000E 00

31	ACCOA	3.2999D-02	1.2111D-15	-3.3000E-02
32	CS	2.4287D-03	4.1254D-17	-2.6000E-02
33	CSOAA	4.5028D-06	-2.0329D-19	-2.6000E-02
34	CSACCOA	1.7572D-02	1.0626D-16	-2.6000E-02
35	CSI	4.1021D-05	-9.5419D-18	-2.6000E-02
36	CSOAAACCOA	4.5088D-06	-2.1684D-19	-2.6000E-02
37	CSI*/	7.6053D-08	-2.3717D-20	-2.6000E-02
38	CSCITCOA	3.9413D-04	4.2934D-17	-2.6000E-02
39	CSCOA	5.3781D-03	-1.3878D-16	-2.6000E-02
40	CIT	2.1790D-02	-5.0931D-15	2.1790E-02
41	CSCIT	1.7699D-04	-4.1633D-17	-2.6000E-02
42	AC	7.1165D-02	9.0206D-16	-7.4000E-02
43	ACCIT	6.4036D-04	-3.4694D-16	-7.4000E-02
44	ACX	9.1750D-04	-9.7145D-17	-7.4000E-02
45	ACA/	1.2867D-04	-4.1633D-17	-7.4000E-02
46	A	8.3621D-04	-1.8041D-16	-1.0000E-03
47	ACIC	1.1563D-03	-4.1633D-16	-7.4000E-02
48	IC	1.3378D-03	-2.8536D-16	1.3378E-03
49	ID	1.4548D-02	-2.2204D-16	-9.2000E-02
50	IDNAD	8.9977D-04	1.9628D-15	-9.2000E-02
51	IDX	1.8135D-06	4.3368D-19	-9.2000E-02
52	IDY	7.2969D-07	-6.5052D-19	-9.2000E-02
53	CO2	2.3939D-05	-6.1257D-18	-1.0000E-04
54	IDNADH	6.9739D-02	-1.9626D-15	-9.2000E-02
55	AOG	7.9880D-06	-1.8974D-18	7.9880E-06
56	ATP	7.8354D-02	-4.7962D-14	-7.1100E-02
57	IDI	6.9197D-03	2.2204D-16	-9.2000E-02
58	KGDH1	3.4406D-03	1.9724D-14	-6.0000E-03
59	KGDH2	6.9261D-08	5.4210D-20	-6.0000E-03
60	KGDH3	1.9216D-08	4.6621D-18	-6.0000E-03
61	KGDH4	2.4521D-04	-3.6429D-17	-6.0000E-03
62	KGDH5	2.3141D-03	-1.9693D-14	-6.0000E-03
63	BICARB	2.9449D 00	2.3939D-03	2.9513E 00
64	ADP	4.6269D 00	4.7740D-14	-4.6330E 00
65	REM	1.0000D 00	0.0000D 00	-1.0000E 00

		FORWARD K	FORWARD FLUX	BACKWARD K	BACKWARD FLUX	NET FLUX
1	2	1.14000D 01	2.0704D-03	6.52000D 01	8.7349D-04	1.1970E-03
3	4	2.48000D 01	2.3983D-03	2.72000D 01	1.2814D-03	1.1970E-03
5	6	4.08040D 04	1.2298D-03	5.47400D 02	3.2868D-05	1.1970E-03
7	8	2.20000D 00	1.0654D-02	1.30000D 02	9.4570D-03	1.1970E-03
9	10	1.10000D 07	4.2313D-01	2.30000D 03	4.2194D-01	1.1970E-03
11	12	4.60000D 04	9.3272D-02	5.50000D 02	9.2875D-02	1.1970E-03
13	14	2.00000D 03	1.9809D-03	8.30000D 03	6.8398D-04	1.1970E-03
15		1.70000D 02	1.1970D-03			
16	17	3.30000D 01	1.3744D-02	1.00000D 03	1.2547D-02	1.1970E-03
18	19	1.00000D 03	9.0815D 01	1.00000D 03	9.0814D 01	1.1970E-03
20	21	1.00000D 03	7.7366D 00	1.00000D 03	7.7348D 00	1.1970E-03
22	23	1.00000D 03	2.0929D 02	2.83000D 02	2.0929D 02	1.1970E-03
24	25	3.00000D 02	1.7056D-01	3.00000D 01	1.6936D-01	1.1970E-03
26	27	3.00000 01	2.9091D-01	3.00000D 02	2.8971D-01	1.1970E-03
28	29	5.50000D 02	7.2866D-02	9.50000D 01	7.1669D-02	1.1970E-03
30	31	1.21000D 03	1.1541D 01	3.60000D 02	1.1540D 01	1.1970E-03
32	33	2.90000D 03	3.3000D 01	4.30000D 01	3.2999D 01	1.1970E-03
34	35	1.75500D 03	1.5082D-04	1.39600D 03	2.2514D-05	1.2830E-04
36	37	1.18100D 03	8.0143D-02	1.66000D 03	7.9874D-02	1.0687E-03
38	39	1.65000D 02	5.3327D-03	1.00000D 03	5.3327D-03	-9.5410E-18
40	41	4.40000D 04	1.4859D-04	1.73000D 04	2.0286D-05	1.2830E-04
42	43	5.70000D 06	1.0912D-03	5.90000D 02	2.2540D-05	1.0687E-03
44	45	1.40000D 05	9.9869D-06	1.00000D 02	9.9869D-06	-2.3717E-20
46	47	5.30000D 03	1.2758D-03	1.00000D 01	7.8825D-05	1.1970E-03
48	49	1.00000D 02	1.1824D-01	1.00000D 02	1.1719D-01	1.0486E-03
50	51	6.60000D 01	1.1824D-02	0.00000D 00	1.1675D-02	1.4838E-04
52	53	0.00000D 00	1.6134D-01	0.00000D 00	1.6029D-01	1.0486E-03
54	55	0.00000D 00	5.3069D-02	0.00000D 00	5.2921D-02	1.4838E-04
56	57	0.00000D 00	8.5288D-01	0.00000D 00	8.5168D-01	1.1970E-03
58	59	0.00000D 00	6.0835D-02	0.00000D 00	5.9638D-02	1.1970E-03
60	61	0.00000D 00	1.1102D 00	0.00000D 00	1.1102D 00	-2.2204E-16
62	63	0.00000D 00	4.3442D-02	0.00000D 00	4.3442D-02	-1.0041E-16

```
   64     65          0.00000D 00       2.66088D 00       0.00000D 00       2.65960 00        1.19708E-03
   66     67          0.00000D 00       4.97220-02        0.00000D 00       4.85250-02        1.19708E-03
   68     69          0.00000D 00       4.38670 00        0.00000D 00       4.38550 00        1.19708E-03
   70     71          0.00000D 00       1.67940-03        0.00000D 00       4.82430-04        1.19708E-03
   72     73          0.00000D 00       1.19700-03        0.00000D 00       6.63790-11 -      1.19708E-03
   74     75          0.00000D 00       1.21130-03        0.00000D 00       1.43390-05 -      1.19708E-03
   76     77          0.00000D 00       1.15070 01        0.00000D 00       1.15060 01        1.19708E-03
   78     79          0.00000D 00       1.02380 00        0.00000D 00       1.02300 00        2.22044E-16
   80     81          0.00000D 00       1.19720-03        0.00000D 00       2.85600-07 -      1.19708E-03
   82     83          0.00000D 00       1.19820-03        0.00000D 00       1.26790-06        1.19708E-03
   84     85          0.00000D 00       7.22710-03        0.00000D 00       6.03810-03        1.19708E-03
   86     87          0.00000D 00       1.44670-01        0.00000D 00       1.43470-01        1.19708E-03
   88     89          0.00000D 00       5.56940 01        0.00000D 00       5.56930 01        1.19708E-03
      90              0.00000D 00       2.39390-03
   91     92          0.00000D 00       9.11120 02        0.00000D 00       9.11120 02        3.59099E-03
   93     94          0.00000D 00       3.30670 01        0.00000D 00       3.30650 01        1.19708E-03
   95     96          0.00000D 00       6.87370-01        0.00000D 00       6.86170-01        1.19708E-03
   97     98          0.00000D 00       4.99990 01        0.00000D 00       5.00010 01       -1.19708E-03
   99    100          0.00000D 00       6.60010 01        0.00000D 00       6.60000 01        1.19708E-03
NUMBER OF DERIVS  398    MATRIX CALCS  4    STEPS  204    A LOCNS  574    SETUPS 4  RESTARTS  0
 TIME =    0.74998750 03
           STOP   ;
NUMBER OF DERIVS  398    MATRIX CALCS  4    STEPS  204    A LOCNS  574    SETUPS 4  RESTARTS  0
 TIME =    0.74998750 03
```

Acknowledgement

I should like to express my most sincere gratitude to Dr. J.H. Ottaway for his considerable and considerate supervision of this project.

I should also like to convey heartfelt thanks to the following people: to Dr. D.K. Apps for many instructive discussions, encouragement and sympathy (when required); to Dr. R.A. Elton for his most necessary guidance in matters statistical; to Helen Scott for her efficient and speedy typing of this thesis; to Cath and Geoff who made work and parties easier to survive; and last, but by no means least to Ross, Mum and Dad who make life and work worthwhile and enjoyable.

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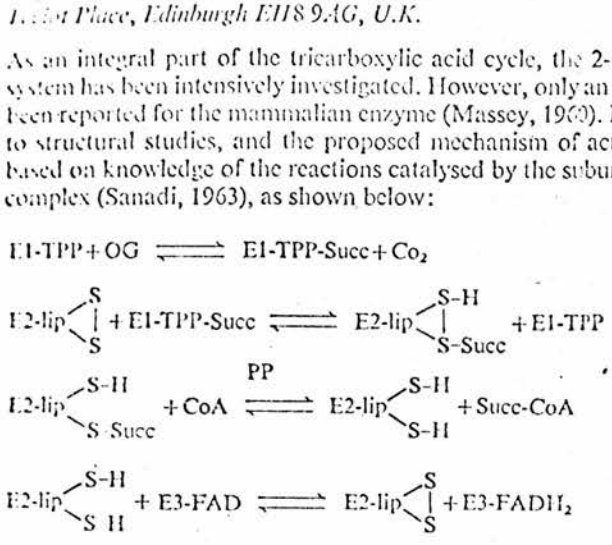
As an integral part of the tricarboxylic acid cycle, the 2-oxoglutarate dehydrogenase system has been intensively investigated. However, only an elementary kinetic study has been reported for the mammalian enzyme (Massey, 1960). Most work has been devoted to structural studies, and the proposed mechanism of action of the system has been based on knowledge of the reactions catalysed by the subunits and cofactors within the complex (Sanadi, 1963), as shown below:



Where TPP represents thiamin pyrophosphate, OG is 2-oxoglutarate, Succ is succinate, lip is lipoate and Succ-CoA is succinyl-CoA (3-carboxypropionyl-CoA). For purposes of a computer simulation of this enzyme system, a more detailed study of kinetics and mechanism was necessary.

To this end, a series of initial-velocity studies was carried out by the Fromm (1967) method. This is a steady-state kinetic approach to the study of the mechanism of action of three substrate enzymic reactions, by which Ping Pong mechanisms can be distinguished from sequential mechanisms. The procedure involves measuring the initial velocity at varying concentrations of one substrate at a series of fixed concentrations of the other two substrates, which are maintained at a constant ratio to each other. The process is repeated for each substrate as the variable one. Lineweaver–Burk plots of the resulting data yield converging lines if a quaternary complex is involved and one or more sets of parallel lines if the mechanism is Ping Pong.

2-Oxoglutarate dehydrogenase was prepared from fresh pig heart by the method of Sanadi (1969). Concentration ranges used in this study were 0.015mM to 0.75mM for 2-oxoglutarate, 0.0025mM to 0.1mM for CoA and 0.01mM to 0.333mM for NAD^+. The system was assayed for NADH production by using an SP. 1800 recording spectrophotometer.

The mechanism proposed by Sanadi (1963), in the notation of Cleland (1963), is a Hexa Uni Ping Pong reaction. According to Fromm's (1967) hypothesis this should yield three sets of parallel lines. This was not found to be the case. Only with varying [2-oxoglutarate] was there a parallel set of plots. The remaining two sets of assays yielded

$$v = \frac{t[A][B][C]}{K_m^* K_I^* [A] + K_m^* [A][B] + K_m^* [A][C] + K_m^* [B][C] + [A][B][C]} \quad (1)$$

where A = 2-oxoglutarate, B = CoA, C = NAD^+.
It was also noticeable that these converging plots were not linear (see Fig. 2*a*).

Attempts were made to fit the experimental data to eqn. (1) by using an optimization package (Davis & Ottaway, 1972). No satisfactory fit could be obtained, however, and this mechanism was discarded.

Another mechanism considered was one where the addition of CoA and NAD^+ is random, as shown in Fig. 1. The initial-rate equation for this cannot easily be expressed in kinetic constant form: it is

$$v = \frac{K_1[A][B][C] + K_2[A][B]^2[C] + K_3[A][B][C]^2}{K_4[A] + K_5[A][B] + K_6[A][C] + K_7[A][B]^2 + K_8[A][B]^2 + K_9[B][C] + K_{10}[B]^2[C] + K_{11}[B][C]^2 + K_{12}[A][B][C] + K_{13}[A][B]^2[C] + K_{14}[A][B][C]^2} \quad (2)$$

where K_1 to K_{14} are complex constants consisting of combinations of rate constants, and A, B and C are as eqn. (1).

The experimental data were fitted to this mechanism as described above. The resulting double-reciprocal plots for CoA and NAD^+ as variable substrate appear as hyperbolic curves. Fig. 2 shows lines fitted by computer optimization for two sets of assays, one with CoA variable, the other with 2-oxoglutarate variable.

The fit to the experimental points is better than for eqn. (1). In particular the general shape of the non-linear curves is obtained (e.g. Fig. 2*a*). However, the overall fit is still not good enough to justify acceptance of this particular mechanism.

One such mechanism which would yield a semi-random mechanism is that in which CoA could bind to the succinyltransferase either before or after the lipoate was re-oxidized. It is of significance in this respect that Sanadi's (1963) mechanism is essentially a pre-steady-state one, and does not specifically consider what might happen when the pseudo-steady-state is established.

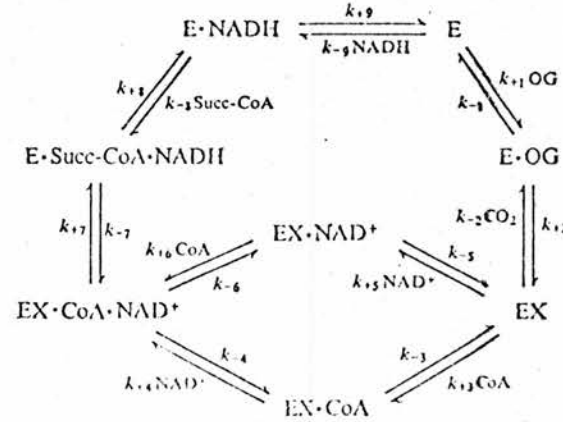


Fig. 1. *Postulated mechanism of action of 2-oxoglutarate dehydrogenase system*

This mechanism has a random order of addition of the substrates CoA and NAD^+. Abbreviations: OG, 2-oxoglutarate; Succ-CoA, succinyl-S-CoA.

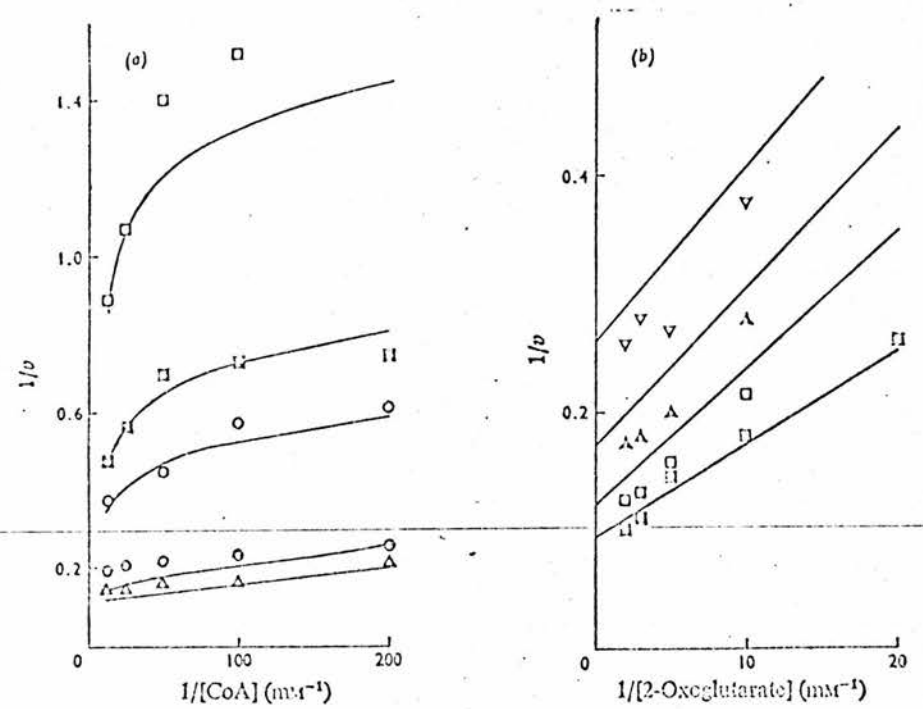


Fig. 2. *Double-reciprocal plots of 2-oxoglutarate dehydrogenase data*

The lines show the best fit of eqn. (2) by computer optimization. All points represent the mean of two measurements. (*a*) CoA is the variable substrate. □, 0.01 mM-NAD^+ 0.015 mM-2-oxoglutarate, ⊡, 0.02 mM-NAD^+, 0.03 mM-oxoglutarate. ○, 0.03 mM-NAD^+, 0.045 mM-oxoglutarate. ◑, 0.133 mM-NAD^+, 0.2 mM-oxoglutarate. △, 0.233 mM-NAD^+, 0.35 mM-oxoglutarate. (*b*) 2-oxoglutarate is the variable substrate. ▽, 0.005 mM-CoA, 0.033 mM-NAD^+. ▲, 0.01 mM-CoA, 0.066 mM-NAD^+. □, 0.02 mM-CoA, 0.133 mM-NAD^+. ⊡, 0.05 mM-CoA, 0.333 mM-NAD^+. *v* is expressed as nmol of NADH produced/min per 0.01 ml of enzyme.

One reason why it would be valuable to establish a mechanism through kinetic studies is the light it may throw on the relationship between structure and function in multienzyme complexes. Tanaka *et al.* (1972) have shown that there are six molecules of the lipoamide dehydrogenase (EC 1.6.4.3) subunit per molecule of lipoate succinyltransferase (succinyl-CoA–dihydrolipoate *S*-succinyltransferase) in the complex. Any mechanism which requires both CoA and NAD^+ to bind to the complex before either succinyl-CoA or NADH are released implies that any one of the lipoamide dehydrogenase subunits must be able to influence the succinyltransferase, e.g. by delaying transfer of the succinyl group or release of succinyl-CoA. It is not obvious how this could be achieved, in view of the complex stoicheiometry.

Financial support from the Medical Research Council is gratefully acknowledged.

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On the Control of Enzyme Pathways

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(*Received* 10 *March* 1974)

Digital computer simulation has been used to study the control of two metabolic pathways, the citric acid cycle in rat heart, and glutathione synthesis in sheep red cells. The latter system was of interest because in a particular breed of sheep the red cell glutathione concentration has been shown to be correlated with a high or a low concentration of the initial enzyme in the pathway, even though the V_{max} for this enzyme is always much larger than that for the second enzyme.

In both instances control lay largely with the first enzyme in the pathway, which is quasi-irreversible, but in the citric acid cycle an important additional control is the rate of production of NADH by isocitric dehydrogenase and oxoglutarate dehydrogenase. This affects the flux through malate dehydrogenase and hence the rate of formation of oxaloacetate, which is needed for the first reaction of the cycle. The other two dehydrogenases are not sensitive to {NADH} in the conditions simulated.

In arriving at this conclusion a modified version of the "elasticity" (effector strength) parameter (Kacser & Burns, 1973) was found to be very useful. The "sensitivity" (control strength) parameter of these authors was not, however, found to be so useful, in part because conditions in which it may be fully applied do not always exist. This was shown to be so for the pathway of glutathione synthesis in red cells.

1. Introduction

he investigations reported in the first part of this paper arose from a quest to simulate the system responsible for the synthesis of glutathione the red cells of the sheep (Young, Nimmo & Hall, 1974; Young, 1975). he conclusions which were drawn from this study were found to be valuable analysing the behaviour of a much more complex system, the citric acid cle, which is also being simulated in this laboratory. Both simulations are ported on in this paper; glutathione synthesis is described first, as only two izymes are involved, and there is only one major conclusion which we think appropriate to draw from the experimental results so far available.

During the investigations, the opportunity was taken of studying th application of the concepts of Sensitivity and Elasticity in to the control c enzyme pathways (Kacser & Burns, 1973; Heinrich & Rapoport, 1974).

GLUTATHIONE SYNTHESIS

Young *et al.* (1974) observed that in the Tasmanian Merino breed c sheep about half the animals have a red cell glutathione concentration c about 2·34 mmole/l cells, while the ratio of V_{max} values for glutamyl-cystein synthase and glutathione synthase (subsequently referred to as GCS and GS respectively), as determined from Lineweaver–Burk plots of initial rates i lysed cell extracts, is about 10 : 1. In the other 50% of animals of this bree the red cell glutathione level is only 0·57 mmol/l, while the GCS/GS rati is 5 : 1. The maximum attainable velocity for the complete synthesis c glutathione, per unit weight of cells, is the same for both strains of sheep The limiting factor must evidently be the maximal activity of glutathion synthase, and thus the difference between the two strains in normal cor ditions appears to be confined to GCS. In view of the similarity in kineti behaviour of the two enzymes in this study, it has been assumed that th molar concentration of GCS enzyme protein is ten times that of GS protei in the first case, and five times greater in the second case. Assuming that th concentration of glutathione is primarily controlled by the rate of synthesi the problem put to us was—"how can the concentration of the final produc of this pathway be altered by variations solely in the concentration of th initiating enzyme, when its catalytic power in the *in vivo* situation is alway greatly in excess of that of the following one?"

2. Methods

Two synthetic enzymes are involved, *glutamyl-cysteine synthase* (EC 6.3.3.2) which catalyses the reaction

$$\text{glutamate} + \text{cysteine} + \text{ATP} \rightarrow \gamma\text{-glu-cys} + \text{ADP} + P_i, \qquad \Delta G_0 = -24 \text{ kJ/m}$$

and *glutathione synthase* (EC. 6.3.2.3), catalysing the reaction

$$\text{glu-cys} + \text{glycine} + \text{ATP} \rightarrow \text{glutathione} + \text{ADP} + P_i, \qquad \Delta G_0 = -24 \text{ kJ/mol}$$

Neither enzyme has been fully studied kinetically, but they have very simil K_m values for substrates and K_i values for products. Both are strong inhibited by ADP, and to a less extent by inorganic phosphate, and th equilibrium constants for the two reactions catalysed are also almost identic For the purposes of this study both enzymes were treated as examples of th sequential Ter–Ter mechanism postulated for glutamine synthase (Meiste

962) as modified by Iqbal & Ottaway (1970). Rate constants for the inter-
nediate steps of the reactions were computed by inserting the experimental
alues for K_m, K_i and K_{eq} into the Haldane and other relationships (Plowman,
972), and then optimizing all these relationships by a SIMPLEX package
Davis & Ottaway, 1972). The behaviour of the complete enzyme system was
imulated by the computer program now called CHEK (Chance & Curtis,
971; Curtis & Chance, 1974).

The problem was chiefly attacked by looking at the steady-state situation.
The substrates (including ATP) were kept constant at concentrations
pproximating those in red cells, as were the concentrations of the products
ADP and P_i. Glutathione is such a poor inhibitor of GS that it matters very
ittle whether its concentration is kept constant or not, but it was usually
ept constant by a dummy removing enzyme. The simulations were run until
he concentration of the intermediate glutamyl-cysteine became constant,
nd the net flux rate was then noted. Runs were repeated at various concen-
rations of GCS while GS was held constant, and vice versa.

We also simulated the initial rates of synthesis of glutathione, since this
pproximated more closely the experimental approach. GCS and GS were
eparately varied, and the concentrations of the substrates were held constant
t physiological levels, but in most runs the products were allowed to
ccumulate unhindered. The system was not in a steady state, since the
oncentration of glu-cys was rising faster than that of glutathione; neverthe-
ss, the conclusions drawn were very similar to those from the steady-state
pproach, and are not shown separately.

3. Results

The results are shown in Figs 1 and 2. The flux (rate of glutathione
nthesis) is almost halved by a reduction of 50% in [GCS] (Fig. 1), and
herefore the postulated physiological control mechanism, by changes in the
oncentration of the first enzyme in the system, is a valid one, although not
uantitatively exact. If the concentration of GCS is increased above normal,
owever, the increase in flux is very small, and beyond a value double the
ormal one it was impossible to keep the system in a steady state.

Figure 2 shows that the flux could also be reduced proportionately by
ecreasing the GS concentration, with GCS held constant. No increase
ould be obtained by increasing the concentration of GS, i.e. the sensitivity†

† Sensitivity of ith enzyme in pathway $(Z_i) = (\mathrm{d}F/F)/(\mathrm{d}[E]_i/[E]_i)$. Elasticity $(e_i) = v/v)/(\mathrm{d}L/L)$, where v is the velocity of the isolated enzyme at the concentration of sub-
ates and products pertaining in the pathway, and L is an effector (which may be a
bstrate or product) (Kacser & Burns, 1973).

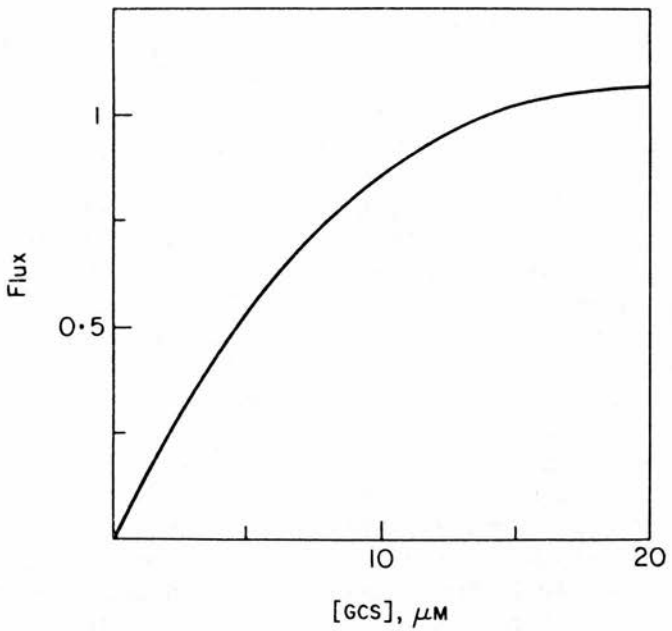

FIG. 1. Flux rate (steady-state rate of glutathione synthesis) in GCS/GS system wit varying concentrations of GCS (glutamyl-cysteine synthetase). [GS](glutathione synthetas held constant at 1 μM.

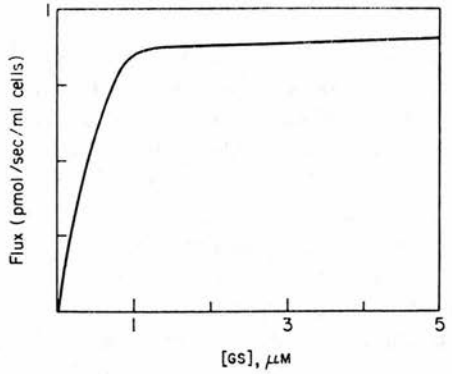

FIG. 2. Flux rate in glutathione synthesis system with varying concentrations of GS. [GCS] held constant at 10 μM.

of the system to this enzyme is zero when it is present in greater than "normal" concentration. The reason which underlies the shape of this curve is discussed later.

Figure 3 represents the Z values plotted against the flux when the concentration of one of the two enzymes was varied while the other was held constant. This is not a satisfactory method of plotting, since it neglects a whole range of mutual concentrations of GCS and GS which would allow a given flux rate, but we have been unable to find a better method.

From these graphs we concluded that the rate of glutathione synthesis might be controlled in red cells, in a downward direction, by a reduction in concentration of either of the two enzymes involved. It was unexpectedly

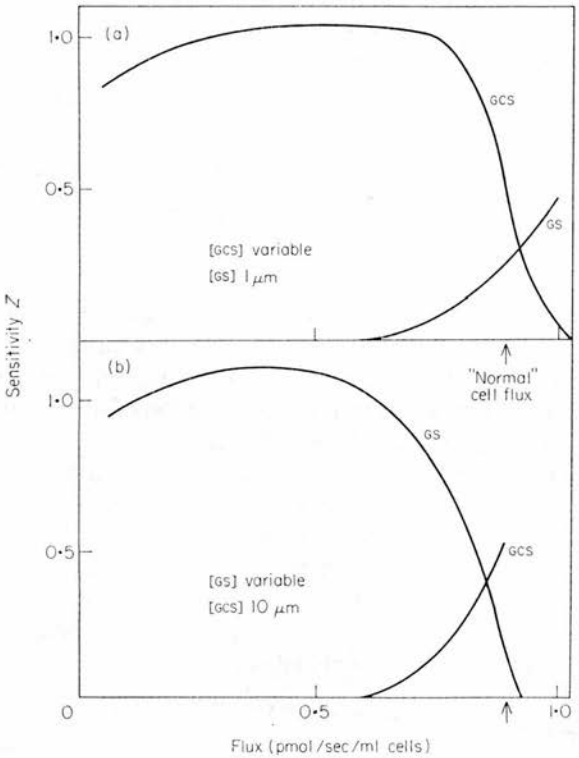

FIG. 3. Sensitivity coefficients for the two enzymes in the glutathione synthesis system plotted against flux rate, (a) with {GS} held constant at 1 μM, (b) with {GCS} held constant at 10 μM.

Note that above the "normal" flux rate (0·09 pmol/sec/ml cells) the sum of the two sensitivity coefficients is no longer equal to 1.

found that the system apparently lies at the limit of controllability in the upward direction; increase in the concentration of either enzyme alone did not affect the flux significantly. It seems clear that the strong control which can be exerted by the first of the two enzymes even though it is present "in excess", depends on the fact that the equilibrium point of the reaction which it catalyses lies very much in the forward direction. Such an observation is not new, but biochemists are often reluctant to give full weight to the importance, in control of biological systems, of an initiating quasi-irreversible enzyme. For example, Rapoport, Heinrich, Jacobasch & Rapoport (1974) established that the control strength of hexokinase is much greater than that of phosphofructokinase in the glycolysis pathway of mammalian erythrocytes, but still regarded the latter enzyme as more important because it is more sensitive to effectors. A similar ambiguity exists with respect to the citric acid cycle, as discussed below.

(A) ANALYSIS OF THE PATHWAY IN TERMS OF 'SENSITIVITY' AND 'ELASTICITY'

An important point relating to the treatment of linear enzyme systems by Kacser & Burns (1973) may be derived from a study of Fig. 3. A feature of the treatment is the postulate that

$$\sum Z_i = 1, \tag{1}$$

that is, the sum of all the fractional changes in flux due to fractional changes in enzyme concentration is unity. However, it is clear from Fig. 3 that at the "normal" level of the two enzymes, $\sum Z_i$ is only 0·7, and the discrepancy becomes even more marked when the concentration of the enzymes, especially GCS, is increased beyond their "normal" level.

It appeared to us that the reason for this must lie in a failure of the system to obey Kacser and Burns' major premis, which is that if the concentration of each of the enzymes in a pathway is increased by a fraction α, the flux will be increased by the same fraction *without any changes in the pool sizes.* This is only true if the concentrations of the various substrates are all below the K_m values for the respective enzymes, so that the differential equation for each intermediate can be simplified to:

$$\mathrm{d}[S_{\mathrm{int}}]/\mathrm{d}t = K - V_{\max} \cdot [S_{\mathrm{int}}]/K_m. \tag{2}$$

The value of S_{int} at infinite time (steady-state), obtained after integrating equation (2) is then

$$[S_{\mathrm{int}}]_\infty = KK_m/V_{\max}. \tag{3}$$

If K, the input rate, is also proportional to some $V_{\max}/K_m$ ratio, and the two K_m's are fixed, the value of $[S_{\mathrm{int}}]_\infty$ is only dependent on the ratio of the

two V_{max}'s (and thus on the ratio of the two enzyme concentrations); it does not depend on their absolute values.

However, if the concentration of S_{int} is high enough for its efflux from its pool to be obligatorily represented by a full Michaelis–Menten expression, the differential equation for S_{int} with respect to t becomes:

$$d[S_{int}]/dt = K - V_{max} \cdot [S_{int}](K_m + [S_{int}]). \quad (4)$$

This would represent the situation in our study, in which the primary substrates were held artificially constant, so that the influx K to the second enzyme was proportional only to the concentration of the first enzyme (GCS). The solution to this equation cannot be made explicit for $[S_{int}]$ (Ottaway & Mowbray, 1973), but there is no doubt that the value of the latter is not independent of the absolute values of K and V_{max}. Thus a fractional change in the concentration of each enzyme no longer leads to a fractional change of equal magnitude in the flux, without change in the pool sizes, and equation (1) no longer holds.

The importance of this in the regulation of the model can be seen from Figs 4 and 5, in which the computed concentration of the intermediate glu-cys is shown for all combinations of [GCS] and [GS] that were investigated. It is clear that over most of these enzyme ranges, [glu-cys] was well above the concentration corresponding to the K_m for GS. The range in

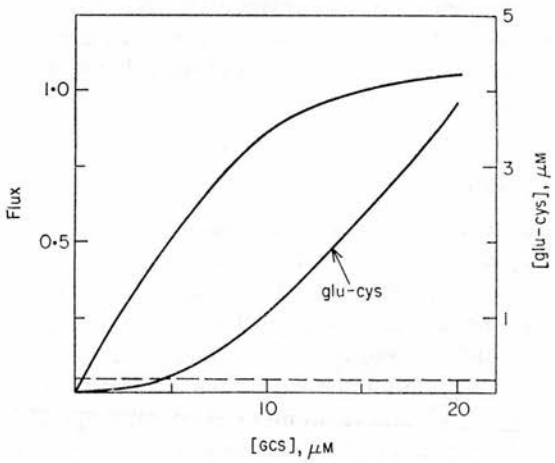

FIG. 4. Flux rate in glutathione system with varying {GCS}, and {GS} held constant cf. Fig. 1). The labelled curve is the concentration of the intermediate glutamyl-cysteine n the steady-state (where this exists). The right-hand ordinate applies to this curve.

The dotted line corresponds to the half-saturation (K_m) concentration for glutathione ynthetase (GS).

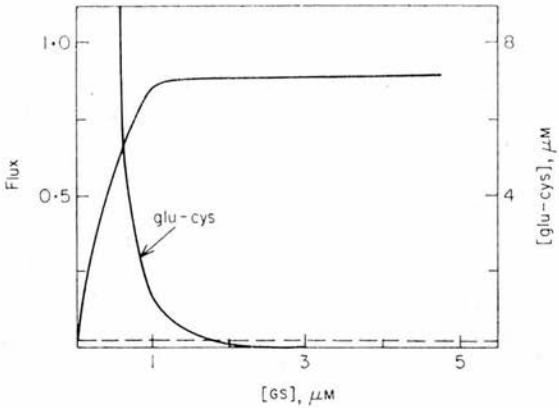

FIG. 5. As for Fig. 4, but {GCS} held constant and {GS} varied (cf. Fig. 2).
Note the inverse relation between {glutamyl-cysteine} and {GS} above 1 μM GS, while the flux rate stays completely constant.

which the underlying premiss of Kacser and Burns was valid can be deduced from Fig. 5, and corresponds to the flat part of the flux curve. We verified by computation that within this range, for a fixed value of [GCS], doubling [GS] reduces [glu-cys] by half, and thus there is no effect on the flux. Figure 4 shows, on the other hand, that if [GCS] is increased with [GS] fixed, the concentration of glu-cys eventually rises beyond the saturation level for glutathione synthase, and at this point, as already mentioned, it was no longer possible to maintain a steady-state during the simulation.

(B) THE CITRIC ACID CYCLE

The "pacemaker" of the cycle

We have already remarked on the strong tendency of biochemists to favour control by the most "responsive" enzyme of a metabolic pathway even when it can be demonstrated that the major control strength lies elsewhere. This is very noticeable with regard to the citric acid cycle; isocitrate dehydrogenase, which is sensitive to several heterotropic effectors (cf Illingworth, 1972), is widely regarded as the "pacemaking" enzyme in the cycle, as for example by Conn & Stumpf (1972), although the importance of the quasi-irreversible reaction catalysed by citrate synthase was pointed out long ago (Krebs & Lowenstein, 1960). The primary role of the latter enzyme emerges very clearly from an analysis of the citric acid cycle by simulation techniques, and the parallel with the simpler glutathione system is very striking. At the same time, the simulation shows that if the activity of citrate

synthase is held constant, control may pass, through a metabolic product of the cycle, to a later stage, but still *not* that catalysed by isocitrate dehydrogenase. We would like briefly to report on these observations; a fuller analysis will be presented elsewhere.

Table 1 shows the Sensitivities of the enzymes of the cycle, in conditions which appear to mimic adequately those obtained in heart mitochondria, in terms of the flux of acetyl units and the concentrations of intermediates (Williamson & Corkey, 1969; Mowbray & Ottaway, 1974). (For details of the enzyme concentrations and parameters used see the Appendix.)

TABLE 1

"Sensitivities" of citric acid cycle enzymes

Enzyme	Sensitivity
Citrate synthase	0·835
Aconitase	0·0
Isocitrate Δ	0·083
Oxoglutarate Δ	0·0
Succinyl thiokinase	0·0
Succinate Δ	0·083
Fumarase	0·0
Malate Δ	0·0
	Σ1·001

Flux rate through cycle, 1·2 nmol/sec/kg mitochondria.

The sensitivity for each enzyme is defined as the fractional change in flux rate through the pathway caused by a small fractional change in the concentration of that enzyme. The sum of all sensitivities should equal unity.

The major component in Table 1 is the sensitivity of citrate synthase, and isocitrate and succinate dehydrogenases play a minor role, although the former was simulated as effectively, and the latter as absolutely, irreversible. It has not so far been possible to find a combination of metabolite concentrations and flux in which the sensitivity of isocitrate dehydrogenase is appreciably larger than that shown in Table 1. Note that the sum of the sensitivities in this model is unity, thus equation (1) holds.

Quasi-irreversible enzymes are usually defined as catalysts of reactions whose equilibrium constants differ from unity by several orders of magnitude. Srere (1972) has pointed out that by this criterion malate dehydrogenase should be more irreversible than citrate synthase, as K_{eq} is larger for the former reaction than for the latter. Nevertheless, in the citric acid cycle malate dehydrogenase is not quasi-irreversible, and the mass action ratio is almost equal to K_{eq}, as the enzyme is being forced to work "backwards". In

agreement with this, altering the concentration of malate dehydrogenase did not alter the flux through the cycle. Thus the term "quasi-irreversible" cannot be used absolutely, but must be applied only with regard to the circumstances. Rolleston (1972) has even made out a case for regarding citrate synthase as an equilibrium enzyme when working within the cycle, although we could find no evidence to substantiate this in our simulations.

(c) THE EFFECT OF NADH CONCENTRATION ON THE FLUX THROUGH THE CYCLE

Interesting results were obtained by applying Kacser and Burns' concept of elasticity to the cycle. In doing this we ignored the fact the summation relationship

$$\sum Z_i e_i = 0 \qquad (5)$$

where Z_i is the sensitivity and e_i the elasticity of the ith enzyme, would not necessarily hold for the values we calculated. This is because the relationship was derived for *all* the enzymes affected by a particular ligand (metabolite), while we wish to limit our investigations, at this time, to the citric acid cycle, without bringing in all the associated pathways by which metabolites (coenzymes) are continuously reconverted so that their concentrations within the cycle proper remain in a steady-state. We also tried the innovation of computing the Elasticities of enzymes *in the cycle*, instead of in isolation (see footnote, p. 59). We did this by allowing the system to reach a steady-state, and then holding the concentrations of all metabolites constant except the one under investigation, by means of "dummy" reactions. The concentration of this metabolite was then altered slightly by means of a "dummy" reaction of its own. The CHEK program allows this to be done very readily by means of differential equations that do not obey the stoichiometric laws of chemistry. This "*in situ* elasticity" may produce finite values for enzymes for which the metabolite under investigation is neither product nor substrate, since the metabolic pathway is no longer in a steady state, but we have found this parameter to be a useful index in locating sensitive reactions.

The metabolite which we studied in this way was NADH, which is a product of three enzymes in the cycle but not a substrate of any. In our simulations the $\{NADH\}/\{NAD^+\}$ ratio, and the concentration of NADH, were kept constant by a "re-oxidation" reaction which was independent of flux through the cycle. Table 2 shows the true and apparent elasticities at "normal" {NADH}, 0·194 μmol/kg mitochondria (based on Williamson & Corkey, 1969), while Table 3 gives the values for {NADH} five times greater than "normal", i.e. 0·97 μmol/kg. (In order to keep all other conditions constant {NAD} was kept at its original level.) It will be seen that the differences between apparent and absolute elasticities are very slight, with the

TABLE 2

"Elasticity" of citric acid cycle enzymes towards NADH

Enzyme	Apparent elasticity	Absolute elasticity
Citrate synthase	−0·41	
Aconitase	−0·02	
Isocitrate Δ	−0·76	−0·82
α-Oxoglutarate Δ	−0·31	−0·37
Succinyl thiokinase	0	
Succinate Δ	0	
Fumarase	−0·23	
Malate Δ	−21·7	−21·5

The values in the first column were obtained by varying {NADH} slightly in a simulation of the complete cycle when all other intermediates were held at their steady-state values. Those in the second column were computed from the forward velocities of each enzyme simulated separately at concentrations of substrates and products identical with those found in the cycle simulations. Fractional change in {NADH}, 1% of "normal" steady-state value, taken as 0·194 μmol/kg mitochondria.

Flux through cycle at steady-state, 1·2 nmol/sec/kg mitochondria.

TABLE 3

"Elasticity" of citric acid cycle enzymes towards NADH

Enzyme	Apparent elasticity	Absolute elasticity
Citrate synthase	−0·08	
Aconitase	−0·15	
Isocitrate Δ	−0·92	−0·94
α-Oxoglutarate Δ	−0·84	−0·81
Succinyl thiokinase	0	
Succinate Δ	0	
Fumarase	−5·24	
Malate Δ	−33·4	−36·5

In this computation {NADH} was held at a value five times greater than normal (= 0·975 μmol/kg mitochondria). {NAD} was kept at its previous value, 0·17 mol/kg. The computations were carried out as for Table 2.

Flux through cycle at steady-state, 0·25 nmol/sec/kg mitochondria.

possible exception of fumarase (Table 3). The only enzyme of the cycle whose velocity is significantly affected by variations in {NADH} is malate dehydrogenase, and we found this also to be true when {NADH} was reduced below "normal" level. Thus in the conditions chosen the flux through isocitrate dehydrogenase and α-oxoglutarate oxidase is only marginally affected by the redox potential of the {NADH}/{NAD^+} couple.

The importance of this finding lies in the flux rates shown in Tables 2 and 3. Increasing the NADH concentration reduced the flux rate proportionately and conversely lowering the {NADH} increased the flux rate (not shown). This effect was mediated through changes in the concentration of oxaloacetate. Since the concentration of malate in the steady-state is much greater than that of the other intermediates (malate and fumarate together made up 85% of the total concentration of all species), changes in the {NADH}–{NAD^+} ratio are reflected almost entirely by changes in {oxaloacetate}. In our simulations {acetyl–CoA} was kept constant, and therefore for a given activity of citrate synthase, the flux through the cycle was dependent almost entirely on the {NADH}/{NAD^+} ratio. This is in accord with the views of Williamson, Olson, Herczeg & Coles (1967). In these simulations the proportion of oxaloacetate bound to various enzyme intermediates was just over 20% of the total in the system. The proportion did not change appreciably with variations in the {NADH}/{NAD^+} ratio, so sequestration of substrate (Sols & Marco, 1971) did not play a part in the control exerted by oxaloacetate.

4. Discussion

We do not imagine that these simulations approach a complete description of reality. Even in the simple system of glutathione synthesis many parameters which would be necessary to predict accurately the concentration of glutathione in the two types of red cell remain unspecified. These include, for example, the rate of destruction of glutathione, and even perhaps the futile hydrolysis of glutamyl–cysteine. A number of variables related to the two synthetic enzymes themselves have not been studied in depth (Nimmo & Young, 1975). However the conclusions reached do not depend on the mechanisms chosen, but only on the ratios of K_m and V_{max} values for the two enzymes as experimentally determined. The main conclusion relating to metabolic control was that the concentrations of the two enzymes *in vivo* are so arranged that the concentration of the intermediate glutamyl–cysteine is just at the upper end of the first-order region of the Michaelis–Menten Curve for the second enzyme. Thus any increase in the concentration of the latter would lower the Glu-Cys concentration proportionately, and the flux through the second enzyme would not alter. Any substantial decrease in the

concentration of the second enzyme, on the other hand, would cause the concentration of the intermediate to rise (Fig. 2) to a saturating level, so that changes in the concentration of the second enzyme would alter the flux rate through the system appreciably. Very soon, however, the situation would be reached in which the second enzyme could not keep up with the rate of formation of intermediate by the first enzyme, and the system could not be maintained in a steady-state. Thus both in the normal and mutant sheep erythrocytes described by Nimmo & Young (1975), control remains firmly with the first enzyme, although it is always present in higher concentration. This is because it catalyses a quasi-irreversible reaction, i.e. one occurring with a large decrease in free energy. We found the quasi-irreversible nature of citrate synthase to be similarly important in control of the citric acid cycle, in agreement with Krebs & Lowenstein (1960). We were not able to see any evidence, in our simulations, that citrate synthase was operating as an equilibrium enzyme, as suggested by Rolleston (1971).

We are particularly conscious that our model of the citric acid cycle is still incomplete. Certainly the cycle does not operate *in vivo* with a fixed $\{NADH\}/\{NAD^+\}$ ratio, a constant acetyl–CoA concentration, and no competition between enzymes for free CoA. These and other limitations were present in our model. At the same time, the system was put together purely from descriptions of the individual enzymes; no attempt was made to tailor maximum velocities or K_m values for any of them, or to make any pre-conditions, for example that citrate synthase might be operating in non-equilibrium conditions. That the cycle operates so realistically is a tribute first of all to the accuracy of a large number of enzymologists, and secondly to the reliability of the CHEK program. The fact that it does operate realistically gives confidence in accepting the findings that it provides. Chief amongst these is the importance of malate and fumarate as carbon reservoirs within the cycle, and of the sensitivity of MDH, in contrast to ICDH and α-oxoglutarate DH, to changes in {NADH}. It has become apparent that the inhibition of citrate synthase by ATP (Hathaway & Atkinson, 1965; Shepherd & Garland, 1966) which was postulated to be important in control of flux rate in the cycle, could not be observed with MgATP (Kosiki & Lee, 1966), which is the form likely to predominate intracellularly (Blair, 1971). The alternative postulate has been made that the mitochondrial oxaloacetate level controls the cycle flux (Williamson, Olson, Herczeg & Coles, 1967). So far as we can judge, this view seems likely to be correct, at least in the restricted conditions of our simulations.

Altering the activity of isocitrate dehydrogenase barely affected the flux through the cycle. This was very interesting, since the enzyme is often referred to as "rate-limiting". The lack of effect could be explained in terms

of the control hierarchy that we have demonstrated for glutathione synthesis, namely, that if a quasi-irreversible reaction comes at the beginning of a pathway, within limits the activities of subsequent enzymes do not affect the flux through the pathway, but only the levels of intermediates. We have no doubt that there are circumstances in which isocitrate dehydrogenase does exert a measure of control, for example in liver tissue when the rate of citrate synthesis may be greater than the rate of citrate oxidation; Randle, Denton & England (1968) have shown that this is even possible in heart tissue if the system is loaded with acetate. However, our simulations suggest that in heart muscle the circumstances must be rather unusual for isocitrate dehydrogenase to become rate-limiting.

Finally, our investigations have helped us to assess the sensitivity and elasticity parameters discussed by Kacser & Burns (1973). The principle of such parameters is not new. It was perhaps first introduced into biochemistry by Higgins (1966), and Heinrich & Rapoport (1974) have defined very similar parameters, which they called "control strength" and "effector strength". We have concluded that the "sensitivity" or "control strength" parameter is very useful for concise description of the important control regions in a metabolic pathway, but is not very useful for analysis, because of the overriding effect of quasi-irreversible reactions, whose importance can be inferred on intuitive grounds and in other ways. Indeed, Rapoport *et al.* (1974), when using Heinrich & Rapoport's definitions to analyse the glycolytic pathway, stated explicitly that the control strengths of "equilibrium" enzymes could be ignored. In addition, we have demonstrated that the postulate that the sum of sensitivity coefficients is unity, which is an attractive feature of Kacser & Burns' treatment, is only true if all the enzymes in the pathway are working in the first-order region of their velocity/substrate curves. We have good reason to think that this is not true for glutathione synthesis in red cells, and other instances may also exist (cf. Rapoport *et al.*, 1974).

On the other hand, we found the concept of elasticity, as we have modified it for use within metabolic pathways, to be most valuable. Without its aid, we would not have identified malate dehydrogenase as the only enzyme at all sensitive to changes of {NADH} within the citric acid cycle. Elasticity could clearly be used to study the importance of other products which come from more than one reaction in an enzyme pathway, and in particular for ADP. We did not use it for this metabolite in the glutathione model because we gave both enzymes the same kinetic properties, and their elasticities with respect to ADP would have been almost identical.

This work was carried out while C.L.M. was holding a Postgraduate Training Grant from the Medical Research Council.

Appendix

Sources of information about kinetics and mechanism of enzymes used in simulation of citric acid cycle in heart mitochondria.

Citrate synthase (EC. 4.1.3.7)

The mechanism was assumed to be Random Bi-bi (Johannson, Måhlen & Patterson, 1973). K_m values (measured for enzyme from rat tissues) from Srere (1971), and V_{max} from Singh, Brooks & Srere (1970). K_{eq} calculated from values given by Stern, Ochoa & Lynen (1952).

The simulation also contained equations for competitive inhibition of acetyl-CoA by succinyl–CoA; values taken from Smith & Williamson (1971).

Aconitase (EC. 4.2.1.3)

The mechanism used was that proposed by Henson & Cleland (1967), and others. K_m values from Thomson, Nance, Bush & Szczepanik (1966), V_{max} from Villafranca & Mildran (1971). Relative V_{max} values for the interconversions between the three substrates from Thomson *et al.* (1966).

Isocitrate dehydrogenase (EC. 1.1.1.41)

The simulation included only the NAD-linked enzyme. This was assumed to be reversible, in order to use the equilibrium constant in determining intermediate rate constants, but the reaction was made quasi-irreversible by assuming a very low affinity constant for CO_2 (1 M). Ratio of $(V_{max})_f/(V_{max})_r$ in the simulation: ca. 300. K_m values for the ADP-activated enzyme from Plaut (1970). V_{max} from Chen & Plaut (1963). K_{eq} calculated from data of Londesborough & Dalziel (1968).

The simulation also contained equations for inhibition of isocitrate dehydrogenase by ATP. K_i values from Plaut (1970).

α-Oxoglutarate dehydrogenase (EC. 1.2.4.2)

The mechanism was assumed to be Hexa-uni ping-pong (Searls & Sanadi, 1960) but work is in progress to verify this. Kinetic values from Massey (1960).

Succinyl thiokinase (EC. 6.2.1.4)

There is no general agreement in the literature on the mechanism for this enzyme; it was assumed to be Bi Uni-Uni Bi Ping-pong. Kinetic constants from Cha & Parks (1965) and K_{eq} from Nishimura & Grinnell (1972).

Succinate dehydrogenase (EC. 1.3.99.1)

The mechanism, kinetic parameters and rate constants were taken from Zeijlemaker, Devartanian, Veeger & Slater (1969).

Fumarase (EC. 4.2.1.2)

Mechanism and rate constants from Alberty & Pierce (1957).

Malate dehydrogenase (EC. 1.1.1.37)

The mechanism was assumed to be Ordered Bi-bi. Rate constants from Raval & Wolfe (1962).

The concentrations of enzymes were taken from, or calculated as in, Srere (1968). The concentrations of substrates and adenine nucleotides were taken from Williamson & Corkey (1965). The total concentration of diphosphopyridine nucleotides was also taken from these authors, but the ratio $\{NADH\}/\{NAD^+\}$ was fixed at 1 : 1.

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Biochem. J. (1977) **161**, 569–581
Printed in Great Britain

Studies on the Mechanism and Kinetics of the 2-Oxoglutarate Dehydrogenase System from Pig Heart

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(*Received* 13 *August* 1976)

1. The kinetic properties of the 2-oxoglutarate dehydrogenase system were investigated. To this end, initial-velocity studies were carried out by the method of Fromm [(1967) *Biochim. Biophys. Acta* **139**, 221–230]. Reciprocal plots of the results did not agree with those expected for the Hexa Uni Ping Pong mechanism previously proposed for the system. 2. The measured initial velocities were fitted to initial-rate equations corresponding to several possible mechanisms by using a computer optimization technique. Statistical analyses performed on the results of the optimization studies indicated that one mechanism was a significantly better fit to the experimental data than the other mechanisms tested. This mechanism is one in which there is a random order of binding of NAD^+ and CoA and release of succinyl-CoA, although the binding of 2-oxoglutarate and release of CO_2 is still given a Ping Pong mechanism, which precedes the binding of the other substrates. These conclusions were supported by NADH-inhibition studies. 3. The usefulness of the method of fitting initial-rate data to rate equations and the applicability of the proposed enzymic mechanism to the enzyme complex are discussed.

The 2-oxoglutarate dehydrogenase complex was first purified from pig heart muscle by Sanadi *et al.* (1952), and their method has been used with occasional slight modifications by most subsequent workers (e.g. Massey, 1960; Smith *et al.*, 1974; see, however, Hirashima, *et al.*, 1967). Sanadi (1963) was also the first to propose a catalytic mechanism for the overall reaction of the enzyme complex on the basis of his investigations of the roles and locations of the cofactors thiamine pyrophosphate, lipoic acid and FAD within the complex, and of the coenzymes, NAD^+ and CoA, which participate in the reaction (Massey, 1960; Sanadi, 1963). This mechanism is shown in Scheme 1.

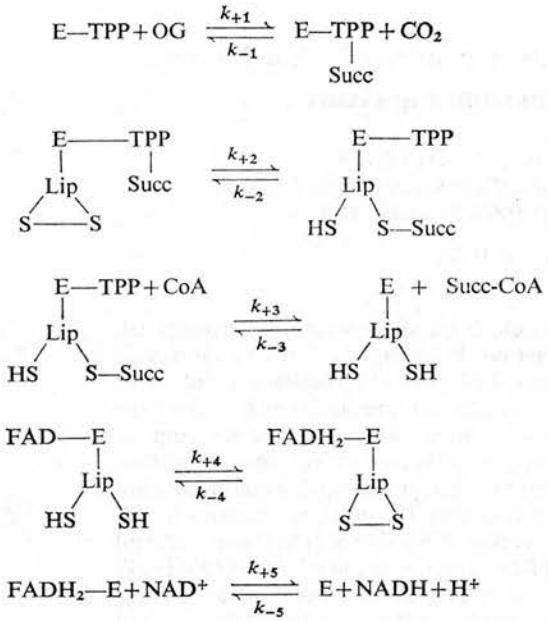
Kinetic studies on the pig heart complex had, until recently, been solely concerned with the measurement of K_m (or K_i) values for one or more substrates (or products) in the presence of saturating concentrations of the other substrates (Massey, 1960; Kanzaki *et al.*, 1969; Johnson & Connelly, 1972; Smith *et al.*, 1974). The values for the kinetic constants that they reported do not agree with each other, but the conditions of assay were different in each case.

To complete our simulation studies of the citric acid cycle (McMinn & Ottaway, 1976), numerical values for the rate constants of the individual steps of the 2-oxoglutarate dehydrogenase reaction were required, and for this purpose it was necessary to check whether the mechanism proposed by Sanadi (1963) for the enzyme complex (Scheme 1) holds. This mechanism is such that the first product (CO_2) is released before the second substrate (CoA) binds, and the second product [succinyl-CoA (3-carboxypropionyl-CoA)] is released before the third substrate (NAD^+) binds. This can be described in Cleland's (1963) terminology as a Hexa Uni Ping Pong mechanism. Establishing the mechanism is particularly important for simulation, since the Sanadi (1963) mechanism predicts that NADH is a competitive inhibitor with respect to NAD^+. We have shown that NADH is an important factor in the control of flux through the citric acid cycle, exerted especially through malate dehydrogenase (McMinn & Ottaway, 1976; Ottaway, 1976).

We needed to simulate the 2-oxoglutarate dehydrogenase accurately enough for its importance as a cycle-regulation point to be assessed.

Hamada *et al.* (1975) attempted to establish the enzymic mechanism from studies of steady-state kinetics. They concluded that their results were compatible with the Hexa Uni Ping Pong mechanism, although at high NAD^+ concentrations there was a divergence from the expected behaviour. They reported that NADH inhibition is competitive with NAD^+, whereas, using very similar substrate concentrations, Smith *et al.* (1974) found that this inhibition was non-competitive. Both groups showed that succinyl-CoA inhibition was competitive with CoA. The results of Smith *et al.* (1974) are not compatible with the Sanadi (1963) mechanism.

The mechanism of three-substrate enzymic reactions can be established by several steady-state



Scheme 1. *Proposed mechanism for 2-oxoglutarate dehydrogenase (Sanadi, 1963)*

Abbreviations used: TPP, thiamin pyrophosphate; Succ, succinic acid; OG, 2-oxoglutaric acid; $LipS_2$, lipoic acid; Succ-CoA, succinyl-CoA. Scheme 1 represents the enzyme complex which contains all three cofactors (TPP, lipoate, FAD). For the sake of clarity only those cofactors involved at each stage of the reaction are shown, although all are present within the complex (E).

kinetic methods (Cleland, 1963; Fromm, 1967; Dalziel, 1969; Fisher *et al.*, 1972). Cleland's (1963) method requires one of the substrates (S_x) to be varied within an assay, while the concentration of a second substrate (S_y) is changed between assays and the third substrate (S_z) is held constant at a saturating concentration. For the investigation to be complete the sets of assays are then carried out with S_z at non-saturating concentrations. The results are plotted in double-reciprocal form. Fromm (1967) suggested a variation of this idea, in which S_x is varied as before, but the concentrations of S_y and S_z are both changed between assays, and the ratio of S_y/S_z is kept constant. Dalziel's (1969) method also involves simultaneously varying the concentrations of all three substrates, but without fixed ratios. With all these methods, product-inhibition studies can give a fuller description of the mechanism, although initial-rate studies (in the absence of inhibitors) for a three-substrate system can be very informative (see Dalziel, 1969).

The method of Fisher *et al.* (1972) requires initial velocities to be measured in both directions of the reaction and is therefore only applicable to enzymic reactions which are readily assayable in both directions.

For our kinetic studies the method of Fromm (1967) was used, for three reasons. (1) The reaction is not easily assayable in the reverse direction. (2) Ping Pong mechanisms can be readily distinguished from sequential mechanisms. (3) Full analysis by initial-rate studies alone does not require as many sets of assays as does Cleland's (1963) method.

Fromm's (1967) analysis predicts that if, the double-reciprocal plots of the results are parallel, then the mechanism is Ping Pong, but if they converge it is sequential. We found this method useful for distinguishing that the addition of 2-oxoglutarate and release of CO_2 is Ping Pong, but it was less useful in analysing the binding order of NAD^+ and CoA, and other methods had to be applied to establish a plausible kinetic mechanism.

A preliminary report of this work has been published (McMinn & Ottaway, 1975).

Materials and Methods

CoA, NAD^+ and 2-oxoglutarate were obtained from Boehringer (Mannheim, Germany). Cysteine hydrochloride was obtained from BDH Ltd. (Poole, Dorset, U.K.). Other chemicals used were of analytical grade. Substrate solutions were prepared fresh each day.

The 2-oxoglutarate dehydrogenase complex was prepared by the method of Sanadi (1969) from fresh pig heart muscle. Concentrations of the final enzyme solutions were between 1.3 and 3 mg/ml, with a specific activity of between 0.75 and 0.85 mol of NADH produced/min per mg of protein at 30°C. The enzyme solutions were stored at −4°C; this produced no appreciable decrease in activity. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Assays of the initial-velocity behaviour were carried out at 30°C in an SP.1800 recording spectrophotometer. Cuvettes were preincubated at 30°C for approx. 5 min before initiating the reaction by the addition of 0.01 ml of enzyme solution. CoA and NAD^+ samples were added just before the preincubation stage to minimize any non-enzymic destruction. All assays were carried out in duplicate. The assay mixtures all contained (in 1 ml) 0.08 M-potassium phosphate buffer (pH 7.2) and 0.4 mM-cysteine hydrochloride (pH 7.0–7.5). The concentration ranges of substrates were: 0.025–0.5 mM-2-oxoglutarate (pH 7.2), 0.005–0.1 mM-CoA and 0.02–0.4 mM-NAD^+ (pH 7.2).

The assay mixtures were set up as described by Fromm (1967) as outlined above. For each set of assays five concentrations of the variable substrate (S_x) and four concentrations of the 'fixed' substrates (S_y and S_z) were used, and each measurement was

ıade in duplicate, i.e. 40 initial velocities per set of ssays, a total of 120 measurements for a complete udy. (In the study reported, only 118 of the measure-ıents were valid, as gross errors in two assays ıvalidated them.) The ratios S_y/S_z used for the three ts of assays were: CoA/NAD^+, 1:8; 2-oxoglutarate/oA, 10:1; 2-oxoglutarate/NAD^+, 5:4. Results were lotted in double-reciprocal form.

Product-inhibition studies with NADH as the ıhibitor were carried out with NAD^+ as the variable ıbstrate and the other two substrates, CoA and oxoglutarate, at saturating and non-saturating ɔncentrations.

The SYMAP program was developed by and is vailable from the Laboratory for Computer raphics and Spatial Analysis, Harvard Center for nvironmental Design Studies, Graduate School of esign, Harvard University, Cambridge, MA 02138, .S.A. Maps were constructed with concentrations two of the substrates as co-ordinates and the odulus of percentage differences between calculated ıd measured velocities as the 'height' or 'density'. ıe chloropleth (area) option, and not the contour ot (Ottaway & Apps, 1972), was used, because it was ɔssible for several observations to have the same -ordinates on a two-dimensional map. A chloro-eth map represents the average value over an area Monkhouse & Wilkinson, 1971).

esults

ualitative analysis of the mechanism

Reciprocal plots of the initial-rate data gave a set linear parallel plots when 2-oxoglutarate was the riable substrate S_x (Fig. 1*a*). However, when CoA NAD^+ was the variable substrate, the plots showed nsiderable non-linearity (Figs. 1*b* and 1*c*). These ults were repeatable with different preparations of enzyme. The only fact that can be certainly duced from these graphs is that 2-oxoglutarate ıds and is decarboxylated, and CO_2 is released ɔm the enzyme complex, before the next substrate ıds. The curvature of the other reciprocal plots kes it impossible to apply Fromm's (1967) rules them.

Bardsley & Childs (1975) have suggested a method analysing non-linear double-reciprocal plots. This essentially an analysis of v against s curves for nts of inflexion and turning points, to gain infor-tion about the degree to which the substrate terms raised in the numerator and denominator of the equation. We found with our data that we could ne to no definite conclusions, because the points nflexion could not be located precisely enough on gainst s plots; very many more data would be ded for this method to be successful. Both more data points for each assay and more replicates of each point measured would be required.

Quantitative analysis of the mechanism

Since no further information could be gained from the data by either Fromm's (1967) analysis or that of Bardsley & Childs (1975), it was decided to use a different approach, that of 'total fit'. The fit of the complete set of initial-velocity measurements to the rates predicted by (steady-state) initial-velocity equations corresponding to several kinetic mechanisms was examined by an optimization procedure as described by Davis & Ottaway (1972). All five mechanisms shown in Fig. 2 were fitted to the experimental data with equivalent initial estimates of the unknown kinetic constants. The best fit was obtained by minimizing the value of R_m, where R_m is defined by eqn. (1):

$$R_m = \sum_{1}^{118} \left(\frac{v_{calc.} - v_{meas.}}{v_{calc.} + v_{meas.}} \right)^2 \quad (1)$$

where $v_{calc.}$ is the initial velocity calculated according to the given rate equation containing the current estimates of the unknown parameters, and $v_{meas.}$ is the corresponding experimental value (Ottaway, 1973).

We found with our initial-rate data that there was a positive correlation between initial velocity and the unsigned errors. This correlation was abolished by the use of the fractional deviations as described, and we verified this, for our own results, graphically. Methods of dealing with more systematic variations have been discussed by Storer *et al.* (1975).

Experience showed that the program called SIMPLEX (see Nelder & Mead, 1965; Davis & Ottaway, 1972) was faster than more sophisticated procedures for large sets of data, and, moreover, convergence to a minimum did not depend on the initial estimates of the parameters.

The five mechanisms shown in Scheme 2 were chosen on the following basis. Mechanism 1 is the Hexa Uni Ping Pong (Sanadi, 1963) mechanism. Although the Fromm (1967) analysis predicts that this is unlikely to be the mechanism of the enzyme, it is useful as a basic model with which other mechanisms can be compared. Mechanism 2 has a sequential binding of CoA and NAD^+. From the information that can be gained from the initial-rate data, this is as plausible a mechanism as the Hexa Uni Ping Pong, and therefore must be included. Mechanisms 4 and 5 both have random binding of CoA and NAD^+ and both would be expected to give non-linear reciprocal plots where CoA or NAD^+ is the variable substrate. Mechanism 3 was suggested by Hamada *et al.* (1975) as a likely explanation of their results at high NAD^+ concentrations. Analysis of our own results by

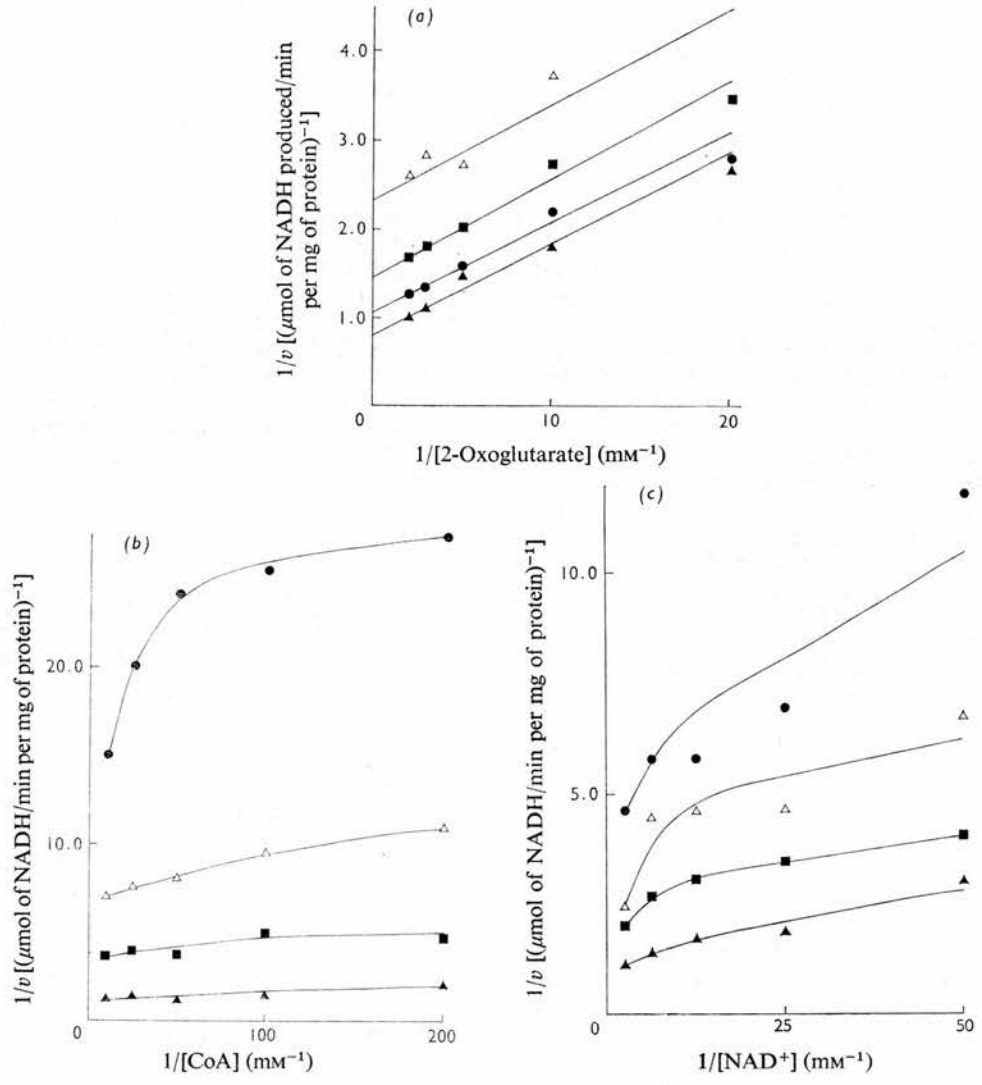
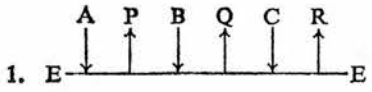


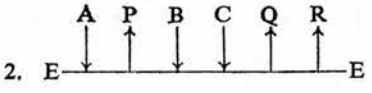
Fig. 1. *Double-reciprocal plots of the results of the initial-rate studies on the 2-oxoglutarate dehydrogenase system* Points shown on the graphs are the average of two measurements. Velocities are expressed as μmol of NADH produced, min per mg of protein. (*a*) 2-Oxoglutarate as the variable substrate. Concentrations of the fixed substrates were NAD^+, 0.333 mM, and CoA, 0.05 mM, (▲); NAD^+, 0.133 mM, and CoA, 0.02 mM (●); NAD^+, 0.066 mM, and CoA, 0.01 mM (■); NAD^+, 0.033 mM, and CoA, 0.005 mM (△). Lines were fitted by computer by Cleland's (1963) method. (*b*) CoA as the variable substrate. Concentrations of the fixed substrates were: 2-oxoglutarate, 0.5 mM, and NAD^+, 0.4 mM (▲) 2-oxoglutarate, 0.1 mM, and NAD^+, 0.08 mM (■); 2-oxoglutarate, 0.05 mM, and NAD^+, 0.04 mM (△); 2-oxoglutarate 0.025 mM, and NAD^+, 0.02 mM (●). Lines were fitted by eye. (*c*) NAD^+ as the variable substrate. Concentrations of the fixed substrates were: 2-oxoglutarate, 0.5 mM, and CoA, 0.05 mM (▲); 2-oxoglutarate, 0.2 mM, and CoA, 0.02 mM (■) 2-oxoglutarate, 0.1 mM, and CoA, 0.01 mM (△); 2-oxoglutarate, 0.05 mM, and CoA, 0.005 mM (●). Lines were fitted by eye.

Fromm's (1967) method indicated that this is highly unlikely to be the mechanism of the reaction in our conditions, but we included it for comparison. It may be that concentrations of NAD^+ at which Hamada *et al.* (1975) observed this phenomenon were much higher than those we used.

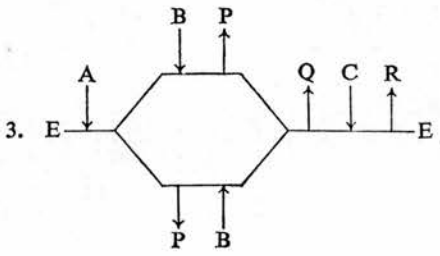
It should be noted that mechanisms 2 and 4 are unlikely to be true for the oxoglutarate dehydrogen

1. 

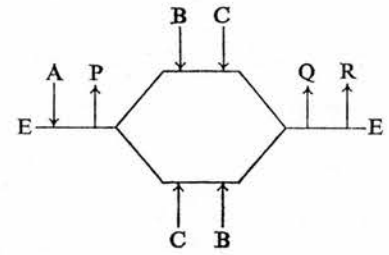
$$v = \frac{V\mathrm{ABC}}{K_m^c\mathrm{AB} + K_m^b\mathrm{AC} + K_m^a\mathrm{BC} + \mathrm{ABC}}$$

2. 

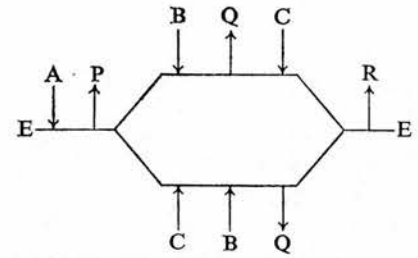
$$v = \frac{V\mathrm{ABC}}{K_m^c K_1^b\mathrm{A} + K_m^c\mathrm{AB} + K_m^b\mathrm{AC} + K_m^a\mathrm{BC} + \mathrm{ABC}}$$

3. 

$$v = \frac{(K_1\mathrm{ABC} + K_2\mathrm{AB^2C})\,E_t}{K_3\mathrm{AB} + K_4\mathrm{AC} + \mathrm{ABC} + K_5\mathrm{BC} + K_6\mathrm{AB^2} + K_7\mathrm{AB^2C} + K_8\mathrm{B^2C}}$$

4. 

$$v = \frac{(K_1\mathrm{ABC} + K_2\mathrm{AB^2C} + K_3\mathrm{ABC^2})\,E_t}{K_4\mathrm{A} + K_5\mathrm{AB} + K_6\mathrm{AC} + K_7\mathrm{AB^2} + K_8\mathrm{AC^2} + K_9\mathrm{BC} + \mathrm{ABC} + K_{10}\mathrm{AB^2C} + K_{11}\mathrm{ABC^2} + K_{12}\mathrm{BC^2} + K_{13}\mathrm{B^2C}}$$

5. 

$$v = \frac{(K_1\mathrm{ABC} + K_2\mathrm{AB^2C} + K_3\mathrm{ABC^2})\,E_t}{K_4\mathrm{AB} + K_5\mathrm{AC} + K_6\mathrm{AB^2} + K_7\mathrm{AC^2} + K_8\mathrm{BC} + K_9\mathrm{B^2C} + K_{10}\mathrm{BC^2} + \mathrm{ABC} + K_{11}\mathrm{AB^2C} + K_{12}\mathrm{ABC^2}}$$

Scheme 2. *Mechanisms fitted to the initial-rate data by SIMPLEX optimization*
Abbreviations: A is [2-oxoglutarate]; B is [CoA]; C is [NAD^+]; P is [CO_2]; Q is [succinyl-CoA]; R is [NADH].

mplex, because it is known that each substrate can act with the complex if an element of the complex in a suitable form (i.e. in the free, succinylated or duced state), in the absence of the other two sub-ates (see Sanadi, 1963). We included them in our alysis because they are kinetically feasible, i.e. we uld not discount them on the basis of our initial-e studies.

The first column in Table 1 shows the values of the nimum differences reached by SIMPLEX for each ing. Although these results indicate that mechanism 5 has a much lower residual than the others, this does not necessarily indicate a better fit to the data, since the values of the minimum differences are not directly comparable, as each equation has a different number of unknown parameters to dispose of.

The fits were examined qualitatively by the SYMAP program. The three maps produced for each mechanism give a good visual indication of the overall fit of an equation to the data. An example of two sets of maps are shown in Fig. 2. These illustrate that mechanism 5 has few areas of high (>20%) error and

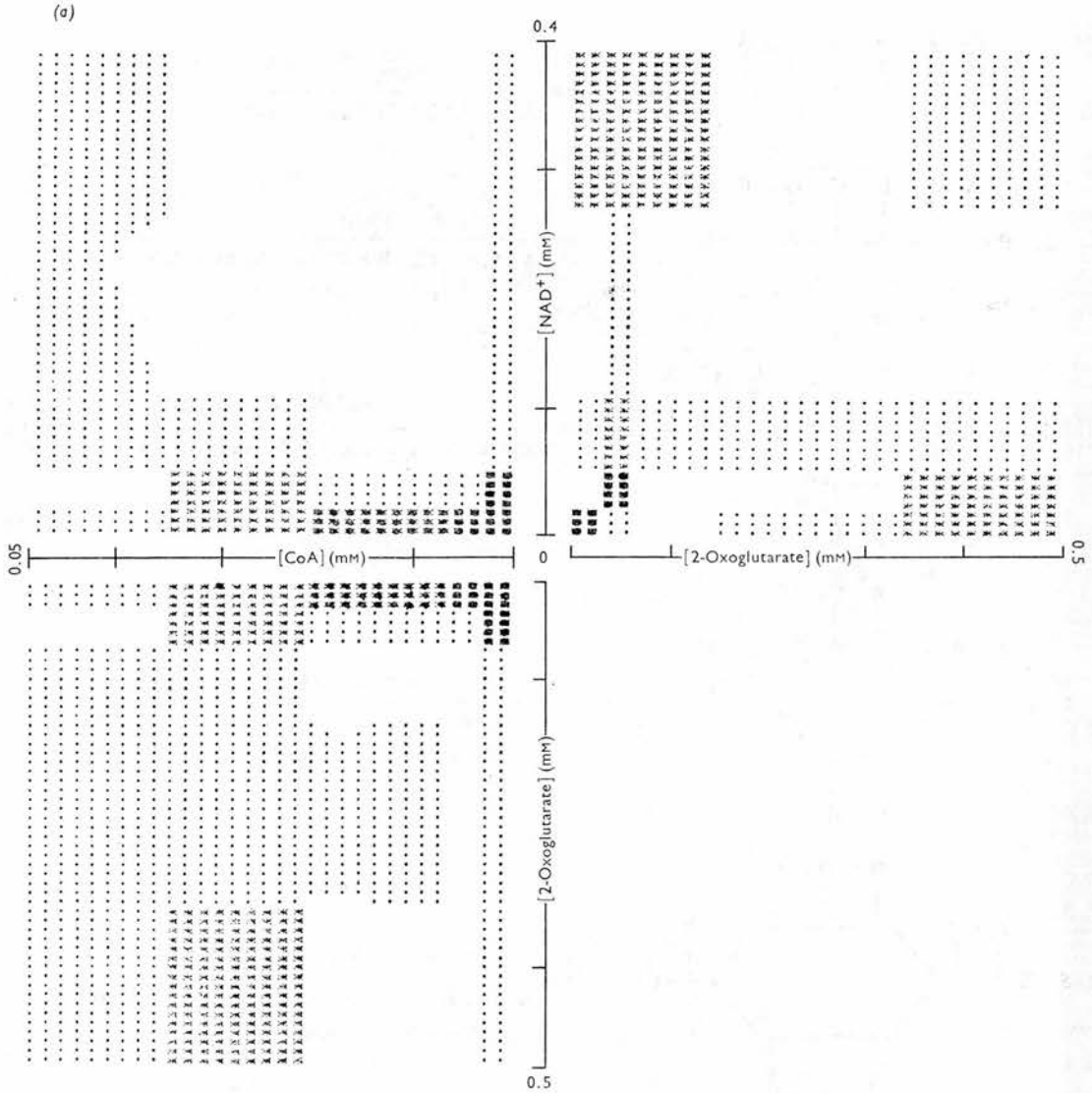
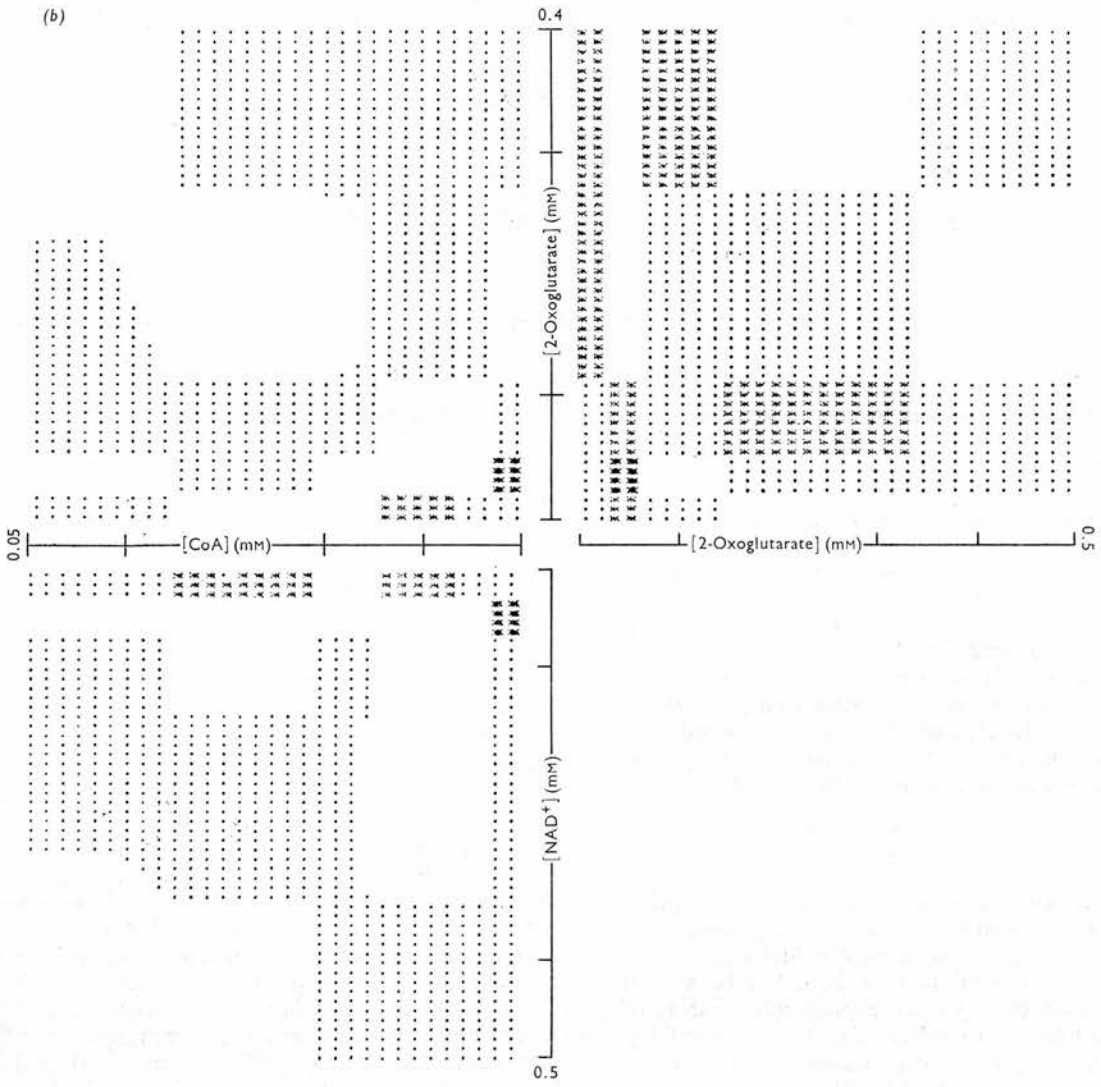


Fig. 2. *Plots of percentage error against substrate concentrati*
Errors are plotted against two of the substrates at one time, as described in the text, resulting in three 'maps', which, wh
the maps are: 0–10% (blank); 10–20% ( ); 20–40% ( ); 40–60% ( ); and 60–100% ( ). (*a*) shows the fit of mecha

overall has a fairly even correspondence between theoretical and experimental values. Mechanism 1, however, shows wide variation in the fitting and has areas of very high (>40%) error. The fit of the other three rate equations was examined similarly and confirmed that mechanism 5 gives the best overall fit of those examined.

Statistical analysis of results

The goodness of fit of the data to the various models was examined statistically in two ways. It should be emphasized that the statistical methods used are some extent empirical, as the theory of non-line least-squares inference has not yet been complete tested (R. A. Elton, personal communicatio However, the need for methods of testing models multi-enzyme systems is so great, in contrast wi single-substrate enzymes for which techniques a now beginning to be agreed (Markus *et al.*, 197 that it has been thought worth while to draw attenti to the approaches outlined below, as they agree wi one another in indicating that one of the mod



for the fit of the initial-rate data to mechanisms 1 *and* 5
put together as above, give a general indication of the fit of the mechanism to the data. The error values appearing on
ism 1 to the data. (*b*) shows the fit of mechanism 5 to the data.

tested fits the data much better than any of the others. What cannot be given at present is any precise estimate of the probability level associated with the observed difference in goodness of fit, as judged by the criteria used.

The first type of analysis we have called 'comparison of models'. It enables a basic model to be tested against a more complex one which is a 'derived version' of the first model (i.e. the equation for the second model contains all the terms in the equation for the first model plus one or more extra terms). Thus mechanism 1 is a basic model from which each of the other four may be derived. Hence we are testing whether the extra terms in the equations corresponding to the complex models (2–5) are contributing to a significantly better fit.

For any given model x, the minimized weighted sum of squares of deviations from the estimated values is R_x (eqn. 1). If the standard deviation of $v_{meas.}$ is assumed to be proportional to the true mean value of

Table 1. *Results of the 'comparison of models' test*
R_m is the computed minimum differences according to eqn. (1) in the text. Mechanisms are numbered as in Scheme 2.

Mechanism	R_m	df	Mechanisms compared	$F_{calc.}$	$F(0.01)$
1	1.798	113			
2	1.723	112	1 < 2	4.875	6.84
3	1.722	109	1 < 3	1.203	3.17
4	1.631	104	2 < 4	0.73	2.55
5	0.858	105	1 < 5	14.38	2.55

v, S.D. $(v_{meas.}) = 2\sigma v$, say, theh R_x is an estimate of $\sigma^2 df(x)$, where the degrees of freedom $df(x)$ are given by: (the number of measured velocities−the number of unknown constants−1).

Suppose that model x is a 'derived version' of model y, involving extra terms, then R_y will have expected value $\sigma^2 df(y)$, and the degree of improvement from y to x resulting from fitting the extra terms will be measured by the difference $R_y - R_x$, with expected value $\sigma^2[df(y) - df(x)]$.

In the perfectly linear case, as for example in adding extra terms to a polynomial to improve the fit to a set of data, R_x and $(R_y - R_x)$ would be independent. If we assume that this is approximately true here and that the data are normally distributed, then:

$$\frac{(R_y - R_x)/[df(y) - df(x)]}{R_x/df(x)}$$

will be approximately distributed as $F[df(y) - df(x), df(x)]$ if both models are true (i.e. if the extra terms are unnecessary). Thus large F values suggest that model y is a better fit than model x. In other words, small values of F, for the appropriate numbers of degrees of freedom, can be used to support the validity of the null hypothesis (that the extra terms did not lead to any improvement in fit). The results of this test are shown in Table 1. The mechanisms compared are those shown in the third column. The large F value for mechanism 5 can be used as a guide to indicate that this is the only model of those tested which gave a better fit to the data than mechanism 1 (Hexa Uni Ping Pong mechanism).

The second statistical test is an analysis of variance. For this test R_e, that part of the deviations caused by experimental error in measuring the initial velocities, was calculated by the formula:

$$R_e = \tfrac{1}{2} \sum_{i=1}^{60} \left(\frac{v_{i1} - v_{i2}}{v_{i1} + v_{i2}} \right)^2 \qquad (2)$$

where v_{i1} and v_{i2} are replicates. (Some 60 measurements were made with each enzyme preparation; see the Materials and Methods section.) This is an unbiased estimate of $\sigma^2 df(e)$, whether or not the model is true. By using this value R_e, and the R_m values as in the previous test, the corresponding mean square (MS) values are calculated by the formulae:

$$MS_m = \frac{(R_m - R_e)}{(df_m - df_e)}$$

and

$$MS_e = \frac{R_e}{df_e}$$

The mean square values are compared by an F test, where:

$$F[(df_m - df_e), df_e] = MS_m/MS_e$$

If the value of F is significant, then the error (deviation) due to the fitting is significantly larger than that due to the measurements; hence the fit to the data is not as good as it could be. If, however, F is not significant, this implies that the error due to the fitting is no greater than the error in the measurements, and such a mechanism can be considered as an acceptable fit to the data. (The limitations on the term 'significant' discussed at the beginning of this section still hold here.)

The results of this test are shown in Table 2. Only mechanism 5 yields a non-significant value for F, which allows us to infer that this mechanism has a high probability of being an acceptable description of the experimental data, in contrast with the other mechanisms tested. However, it must be pointed out that statistical tests cannot show that any particular mechanism is a unique description of the data.

Inhibition studies with NADH

Double-reciprocal plots of the NADH-inhibition results are shown in Fig. 3. Where CoA and 2-oxoglutarate are saturating the patterns indicate a mixed-type inhibition and this also seems to be the inhibition type when both of these substrates are

Table 2. *Results of the 'analysis of variance' test*

Mechanisms are numbered as in Scheme 2. R_m, df_m, R_e, df_e, MS_m, MS_e and F are calculated as described in the text. The theoretical value of $F_{50,60}$ is 1.88. This is taken from *Documenta Geigy*.

Mechanism	R_m	df	R_e	df	MS_m	MS_e	F
1	1.798	113	0.4064	58	0.0253	0.00697	3.63
2	1.723	112	0.4064	58	0.0244	0.00697	3.50
3	1.722	109	0.4064	58	0.0258	0.00697	3.70
4	1.631	104	0.4064	58	0.0266	0.00697	3.82
5	0.858	105	0.4064	58	0.0096	0.00697	1.37

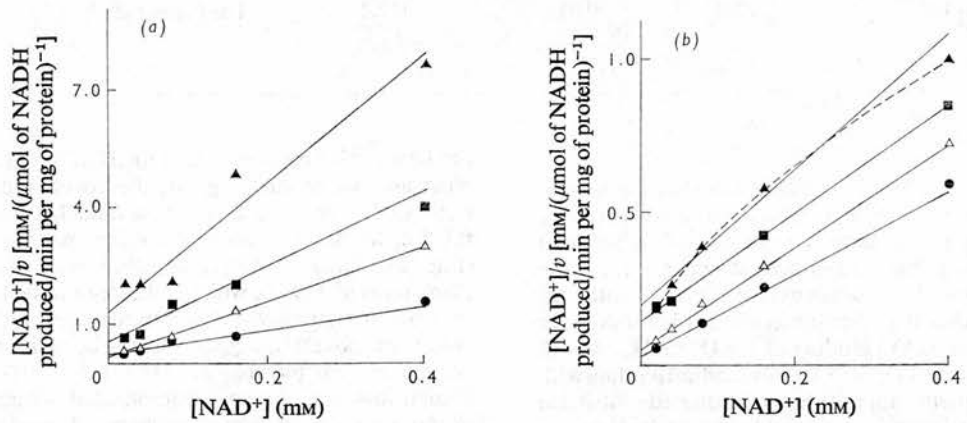


Fig. 3. *s/v against s plots of the NADH-inhibition data where NAD+ is the variable substrate*

NAD+ was the variable substrate. NADH concentrations were 0.0 (●), 0.01 mM (△), 0.02 mM (■) and 0.05 mM (▲). Concentrations of the fixed substrates were (*a*) 2-oxoglutarate, 0.05 mM, and CoA, 0.005 mM (both non-saturating), and (*b*) 2-oxoglutarate, 0.5 mM, and CoA, 0.05 mM (both saturating). Lines were fitted by computer by Cleland's (1963) method. The curved line in (*b*) was fitted by eye. Initial velocities are expressed in μmol of NADH/min per mg of protein.

Table 3. *Values for the optimized constants of the rate equation for mechanism 5*

The constants are numbered as in mechanism 5 in Scheme 2. Values were calculated in μmol of NADH produced/μmol of enzyme, assuming a mol.wt. of 7×10^6 for the 2-oxoglutarate dehydrogenase complex.

Constant	Value
K_1	$21.6\,s^{-1}$
K_2	$467.0\,s^{-1}\cdot mM^{-1}$
K_3	$1703.0\,s^{-1}\cdot mM^{-1}$
K_4	0.75×10^{-10} mM
K_5	0.5×10^{-5} mM
K_6	0.44
K_7	0.17
K_8	0.62 mM
K_9	0.20
K_{10}	5.18
K_{11}	$17.8\,mM^{-1}$
K_{12}	$27.1\,mM^{-1}$

non-saturating. We have fitted straight lines to the data, but it should be remembered that these may well be non-linear plots (see Fig. 3*b*). Mechanism 5 predicts that the NADH-inhibition data should yield non-linear plots; however, at the substrate concentrations used, the deviation from linearity is very slight.

Values of kinetic constants

The values of the complex constants for mechanism 5 found by optimization, which will be used in subsequent simulation studies, are given in Table 3. K_m values have no meaning for rate equations as complex as this, but the 'S_{50}' value for each of the substrates was computed from the values in Table 3 by setting the other two substrates at 'saturating' concentrations. Table 4 shows our computed S_{50} values along with the values of K_m for the various substrates which have been reported by previous workers.

Table 4. *Comparison of kinetic constants and S_{50} values for the pig heart 2-oxoglutarate dehydrogenase*
S_{50} values are calculated as described in the text. The S_{50} value given here is the lowest concentration of CoA that will give half-maximal velocity. Mechanism 5 predicts that CoA will exhibit substrate inhibition, so there is more than one concentration of CoA that can give half-maximal velocity. OG, 2-Oxoglutaric acid.

K_m^{OG} (mM)	K_m^{CoA} (mM)	$K_m^{NAD^+}$ (mM)	Conditions	Reference
0.013	0.0001	0.0045	pH7.4 25°C	Massey (1960)
0.22	0.025	0.05	pH7.5 25°C	Hamada *et al.* (1975)
—	0.0027	0.021	pH7.2 22°C	Smith *et al.* (1974)
S_{50}OG (mM)	S_{50}CoA (mM)	$S_{50}NAD^+$ (mM)		
0.2	0.0035	0.05	pH7.2 30°C	The present study

Discussion

The work reported here indicates that the kinetic mechanism of the 2-oxoglutarate dehydrogenase complex is not a simple sequence of substituted enzyme (Ping Pong) reactions. It appears from the statistical significance uncovered by our 'total fit' procedure that the kinetic mechanism is random, at least with respect to binding of NAD^+ and CoA, and the release of succinyl-CoA. This random binding with the consequent appearance of squared substrate terms in the denominator would account for the non-linearity of the reciprocal plots (Fig. 1). It would also account for the non-competitive inhibition of NAD^+ by NADH reported by Smith *et al.* (1974), and our own finding of a mixed inhibition by this product (Fig. 3).

It is possible that the non-linearity of the reciprocal plots could be due to homotropic allosteric effects, but we have rejected this possibility, because there is at present no evidence for allosteric behaviour of the 2-oxoglutarate complex, in contrast with the *Escherichia coli* pyruvate dehydrogenase complex (Shepherd & Hammes, 1976), for which binding of acetyl-CoA at sites other than catalytic sites has been established.

It must be borne in mind, however, that the mechanism that we have proposed for the reaction may be the simplest one which gives an acceptable fit to our data. We have assumed, in constructing the models shown in Scheme 2, that the one part of the overall reaction which does seem certain to involve an ordered substitution step, the decarboxylation of oxoglutarate, precedes the other two stages, because in the steady state neither the transfer of the succinyl residue through the lipoate succinyltransferase subunit to CoA, nor the transfer of 2H through the transferase and lipoamide dehydrogenase subunits to NAD^+, can occur before the oxoglutarate has been decarboxylated. We have also assumed that release of NADH is the last step in the process, but this need not be so. It is noteworthy that the NADH-inhibitic plots are not simple (Fig. 3); moreover, the distr bution of error, even in the best model, is worse i relation to NAD^+ than to the other two substrat (Fig. 2). These findings, together with those Hamada *et al.* (1975), which indicate that the bindir of CoA (or succinyl-CoA) retards the binding NAD^+ (or NADH), suggest that the behaviour of tl enzyme system towards NAD^+ (or NADH) is n straightforward. A more complicated sequence intramolecular transfers, perhaps dependent c substrate concentrations, could be envisaged whic could describe the sequence of events at this point the reaction more accurately.

We do not think, however, that we could prove th more complex models give a better fit. The F test already significant for model 5 at the 1% level probability; other models might give an equal significant result, but there would be no criterion f choosing between them. It is theoretically possib that the 'comparison of models' test could give positive decision, but, since any more complex mod- than those we have suggested would involve eve more parameters in the rate equation the reductic in residual error by the optimization program wou have to be very considerable indeed for statistic significance to be reached. It does not seem like that this would happen, particularly in view of tl difficulty, touched on below, of assuming that 'global minimum' has been reached. We think that tl limit of model building on the basis of steady-sta measurements has been reached, and that other lin of approach are required to establish more precis ly the dynamics of the reactions catalysed by th- complex.

We are nevertheless gratified at the progress whi we were able to make by using 'total fit'. When became clear that the conventional double-reciproc plots were not linear, analysis of the mechanism

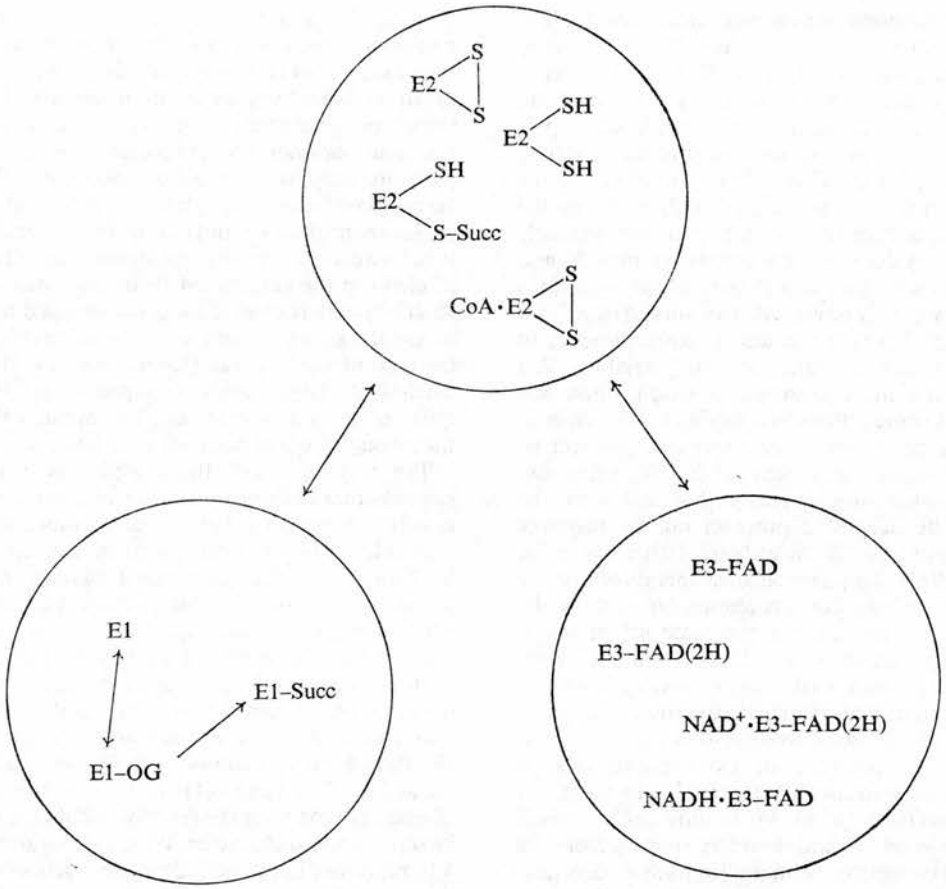
established methods became very unrewarding. The usefulness of 'total fit' was considerably enhanced by applying statistical tests, even if they rest on a somewhat insecure basis, otherwise the decision whether a particular optimization minimum represented a 'satisfactory' fit depended almost entirely on aesthetic judgment about the distribution of error on plots such as those shown in Fig. 2. Although the 'comparison of models' test is particularly valuable for preliminary decisions about the fit of models, and is very convenient for use with enzyme rate equations because they can all be regarded as 'special cases' of a basic model, that is the equation corresponding to the basic model contains all the variables that appear in the more complex equation, there are nevertheless some pitfalls in applying it. The chief of these is that no optimization minimum can ever be guaranteed to be the 'global minimum', i.e. it can never be stated unequivocally that it lies at the centre of the deepest depression on the response surface (Beveridge & Schechter, 1970; Davis & Ottaway, 1972). To guard against this difficulty, we started the optimization procedure for each model from as nearly as possible the same set of initial estimates of parameter values. It is obvious, however, that, if the equation pertaining to model y has more parameters than that of model x, some of the parameters of y cannot be equivalent to any of the 'x' set. Moreover, the presence of extra parameters in equation y may mean that the optimum values of some of those in the 'y' set may be different from their equivalents in set 'x', and therefore starting from the same initial parameter estimates for both models may not in fact be giving set 'y' an equally good opportunity to reach its minimum. There is no solution to this difficulty, and we circumvented it only by choosing several sets of initial values, letting the computer program reach a minimum for each model with each set, and comparing the lowest minima reached for each model. In doing this it was very convenient to use a very robust optimization program such as SIMPLEX, which will reach a minimum from almost any starting co-ordinates, even if it sometimes does so rather slowly, in preference to more sophisticated programs that need good initial estimates. Programs that require the parameters to be scaled internally to have about the same numerical magnitude are particularly difficult to use in the way we have suggested (Davis & Ottaway, 1972).

Although it has been known for some time that the 2-oxoglutarate dehydrogenase complex, like the pyruvate dehydrogenase complex, contains a very large number of peptide chains, and that the three functional groups are attached to three different kinds of protein (Reed, 1966; Koike *et al.*, 1971), the relation between the molecular architecture and the kinetic mechanism has not hitherto been discussed. The reaction is still written as if only three peptide chains are involved, one for each of the partial reactions (Scheme 1; see Hamada *et al.*, 1975). One apparent difficulty is that the numbers of chains of the three types within the complex are not equal; although the values are not known so exactly as they are for pyruvate dehydrogenase, the proportions of the decarboxylase/trans-succinylase/lipoate dehydrogenase chains are probably about 6:1:6 (Tanaka *et al.*, 1972). It is, however, the average residence time of each type of chain in the substituted form that determines its reactivity with respect to the other two, and therefore, unless the structural role of the trans-succinylase as the core of the complex (Koike *et al.*, 1971) is overwhelmingly large, one may suppose that the three types of subunit are present in amounts that give them roughly equal catalytic effectiveness.

The way in which the complex is constructed suggests that each protein chain of a particular type is within range of a few, certainly more than one, molecules of the next catalyst in the sequence shown in Scheme 1. The expression 'within range' is deliberately vague, because it is not clear at present whether physical contact of the active sites is necessary for transfer to take place between the subunits; evidence suggests that, so far as the *E. coli* pyruvate dehydrogenase complex is concerned, the lipoate 'swinging arm' is out of reach of the NAD^+-binding site (Shepherd & Hammes, 1976). We suggest that the ability of each subunit to react with more than one of another type gives the reaction within the complex its semi-random character. We may consider Scheme 3 to represent the three independent sets of subunits that are able to react with each other. Each set contains protein chains both in the substituted and in the normal states.

We have already pointed out that transfer of succinyl and 2H from a reactive site of the set 1 chains is a necessary precursor of subsequent reactions. If the complex as a whole is not working at maximum activity, it is not, however, necessary that the oxidized lipoate chain that accepts the succinyl residue is precisely the one which accepted the previous residue. The latter could remain in the reduced state for some time before being reoxidized, so long as there remains a pool of oxidized lipoate sites in set 2 capable of continuously accepting succinyl residues from a decarboxylase site. Similarly, the FAD of a chain in set 3 could conceivably remain reduced for a finite period of time, and another, within working range of the same trans-succinylase unit, could accept the next pair of oncoming hydrogens.

Thus, on the average, at moderate rates of catalysis, the sequence of events beyond the release of CO_2 would appear to be random. As the complex is forced nearer its saturating throughput, however, the mechanism would become more strictly sequen-



Scheme 3. *Representation of the 2-oxoglutarate dehydrogenase complex showing possible relationship between structure and random-binding kinetics*

This scheme shows some of the species of the three subunits of oxoglutarate dehydrogenase which could co-exist within one molecule of the complex during the catalytic process. Note that for E1 (the dehydrogenase) there is an obligatory reaction sequence for every subunit, whereas for the other two subunits (E2 and E3) this is not necessarily so. For further explanation, see the Discussion section. For explanation of abbreviations, see legend of Scheme 1.

tial, because there would no longer be a pool of unused prosthetic groups within sets 2 and 3. Finally, each succinyl group would have to 'queue up' on the decarboxylase subunit until a lipoate residue became available, and this could only happen when the residue that had been reduced immediately beforehand had been reoxidized by a subunit in set 3. The fact that the mechanism is also Ping Pong in type depends on the fact that the substituted chains are not able to revert to their normal state until the acyl residue has been transferred to another acceptor. This restriction does not hold for E3, and the kinetics with respect to NAD^+ may be slightly different from that for the other two components of the complex.

A proposal of this type explains why the previous workers on the enzyme, who used saturating concentrations of the fixed substrates, reported the mechanism to be Hexa Uni Ping Pong in character (Massey, 1960; Reed, 1966; Hamada *et al.*, 1975). It was not until the rate of reaction began to be measured at non-saturating concentrations of all three substrates that the possibility of random transfer between components within the complex could become manifest.

We are grateful to Dr. R. Elton of the department of Medical Computing and Statistics, Edinburgh University, for much helpful advice on the statistical procedures. This work was carried out during the tenure of a Postgraduate Training Grant from the M.R.C. by C. L. McM.

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